

## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner  
 US Department of Commerce  
 United States Patent and Trademark  
 Office, PCT  
 2011 South Clark Place Room  
 CP2/5C24  
 Arlington, VA 22202  
 ETATS-UNIS D'AMERIQUE  
 in its capacity as elected Office

Date of mailing (day/month/year) 12 February 2001 (12.02.01)	
International application No. PCT/EP00/05457	Applicant's or agent's file reference 0099217-WSmi
International filing date (day/month/year) 14 June 2000 (14.06.00)	Priority date (day/month/year) 22 June 1999 (22.06.99)
Applicant DEN DAAS, Izaak et al	

1. The designated Office is hereby notified of its election made:

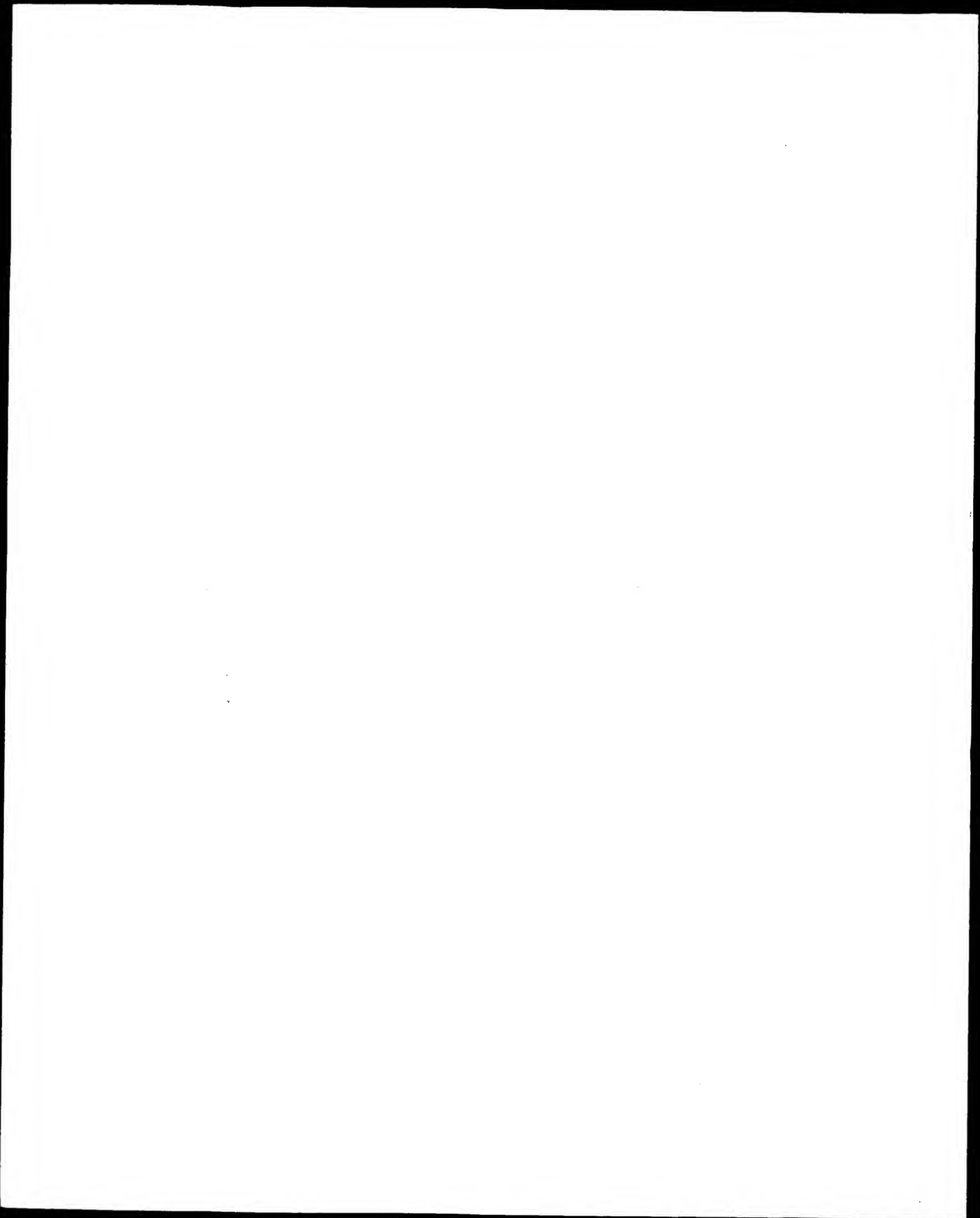
☒ in the demand filed with the International Preliminary Examining Authority on:  
 13 December 2000 (13.12.00)

☐ in a notice effecting later election filed with the International Bureau on:  
 \_\_\_\_\_

2. The election ☒ was  
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer S. Mafla Telephone No.: (41-22) 338.83.38
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REC'D 14 SEP 2001


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## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 0099217-WSmi		<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP00/05457	International filing date (day/month/year) 14/06/2000	Priority date (day/month/year) 22/06/1999	
International Patent Classification (IPC) or national classification and IPC C12N15/12			
Applicant MERCK PATENT GMBH et al			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 4 sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <ul style="list-style-type: none"><li>I <input checked="" type="checkbox"/> Basis of the report</li><li>II <input type="checkbox"/> Priority</li><li>III <input type="checkbox"/> Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</li><li>IV <input type="checkbox"/> Lack of unity of invention</li><li>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</li><li>VI <input type="checkbox"/> Certain documents cited</li><li>VII <input type="checkbox"/> Certain defects in the international application</li><li>VIII <input type="checkbox"/> Certain observations on the international application</li></ul>			
Date of submission of the demand  13/12/2000		Date of completion of this report  12.09.2001	
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465		Authorized officer  Grosskopf, R  Telephone No. +49 89 2399 8714	







# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/05457

## I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

### Description, pages:

1-37 as originally filed

### Claims, No.:

1-11 as originally filed

### Drawings, No.:

1-6 as originally filed

### Sequence listing part of the description, pages:

1-3, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP00/05457

- ☐ the description,      pages:  
☐ the claims,          Nos.:  
☐ the drawings,        sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims
	No:	Claims 1-8
Inventive step (IS)	Yes:	Claims 1-8
	No:	Claims 9-11
Industrial applicability (IA)	Yes:	Claims 1-11
	No:	Claims

- 2. Citations and explanations**  
**see separate sheet**



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/EP00/05457

**Ad item V:**

The quoted documents are:

(1) MIYAMOTO HIROSHI ET AL: "Molecular cloning of a novel mRNA sequence expressed in cleavage stage mouse embryos." MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 34, no. 1, 1993, pages 1-7,

(2) DATABASE EMBL [Online] Accession number AF151824, 1 June 1999 (1999-06-01) LAI C.-H. ET AL.: "Identification of Novel Human Genes Evolutionarily Conserved in Caenorhabditis elegans by Comparative Proteomics"

Both D1 and D2 describe proteins and the corresponding DNAs which are identical (see protein of D2) or nearly identical (homologies of 99.9% for the DNA of D2 and 98.8% and 92.4%, respectively for the protein and the DNA of D1).

Therefore none of the product claims fulfils the requirements of articles 33.2. and/or 33.3 (Claims 1 to 8 lack novelty and claims 9 and 10 are obvious over D1 and/or D2).

The same applies for the general method of Claim 11 which cannot make any contribution to a possible inventive activity.



NIL

# VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT FÜR DEM GEBIET DES PATENTWESSENS

## PCT

### INTERNATIONALER RECHERCHENBERICHT

(Artikel 18 sowie Regeln 43 und 44 PCT)

Aktenzeichen des Anmelders oder Anwalts <b>0099217-WSmi</b>	<b>WEITERES VORGEHEN</b> siehe Mitteilung über die Übermittlung des internationalen Recherchenberichts (Formblatt PCT/ISA/220) sowie, soweit zutreffend, nachstehender Punkt 5
Internationales Aktenzeichen <b>PCT/EP 00/ 05457</b>	Internationales Anmeldedatum (Tag/Monat/Jahr) <b>14/06/2000</b>
	(Frühestes) Prioritätsdatum (Tag/Monat/Jahr) <b>22/06/1999</b>
Anmelder  <b>MERCK PATENT GMBH.</b>	

Dieser internationale Recherchenbericht wurde von der Internationalen Recherchenbehörde erstellt und wird dem Anmelder gemäß Artikel 18 übermittelt. Eine Kopie wird dem Internationalen Büro übermittelt.

Dieser internationale Recherchenbericht umfaßt insgesamt 3 Blätter.

☒ Darüber hinaus liegt ihm jeweils eine Kopie der in diesem Bericht genannten Unterlagen zum Stand der Technik bei.

#### 1. Grundlage des Berichts

- a. Hinsichtlich der **Sprache** ist die internationale Recherche auf der Grundlage der internationalen Anmeldung in der Sprache durchgeführt worden, in der sie eingereicht wurde, sofern unter diesem Punkt nichts anderes angegeben ist.

☐ Die internationale Recherche ist auf der Grundlage einer bei der Behörde eingereichten Übersetzung der internationalen Anmeldung (Regel 23.1 b)) durchgeführt worden.

- b. Hinsichtlich der in der internationalen Anmeldung offenbarten **Nucleotid- und/oder Aminosäuresequenz** ist die internationale Recherche auf der Grundlage des Sequenzprotokolls durchgeführt worden, das

☒ in der internationalen Anmeldung in schriftlicher Form enthalten ist.

☒ zusammen mit der internationalen Anmeldung in computerlesbarer Form eingereicht worden ist.

☐ bei der Behörde nachträglich in schriftlicher Form eingereicht worden ist.

☐ bei der Behörde nachträglich in computerlesbarer Form eingereicht worden ist.

☐ Die Erklärung, daß das nachträglich eingereichte schriftliche Sequenzprotokoll nicht über den Offenbarungsgehalt der internationalen Anmeldung im Anmeldezeitpunkt hinausgeht, wurde vorgelegt.

☐ Die Erklärung, daß die in computerlesbarer Form erfaßten Informationen dem schriftlichen Sequenzprotokoll entsprechen, wurde vorgelegt.

2. ☐ Bestimmte Ansprüche haben sich als nicht recherchierbar erwiesen (siehe Feld I).

3. ☐ Mangelnde Einheitlichkeit der Erfindung (siehe Feld II).

#### 4. Hinsichtlich der Bezeichnung der Erfindung

☒ wird der vom Anmelder eingereichte Wortlaut genehmigt.

☐ wurde der Wortlaut von der Behörde wie folgt festgesetzt:

#### 5. Hinsichtlich der Zusammenfassung

☒ wird der vom Anmelder eingereichte Wortlaut genehmigt.

☐ wurde der Wortlaut nach Regel 38.2b) in der in Feld III angegebenen Fassung von der Behörde festgesetzt. Der Anmelder kann der Behörde innerhalb eines Monats nach dem Datum der Absendung dieses internationalen Recherchenberichts eine Stellungnahme vorlegen.

6. Folgende Abbildung der **Zeichnungen** ist mit der Zusammenfassung zu veröffentlichen: Abb. Nr. \_\_\_\_\_

☐ wie vom Anmelder vorgeschlagen

☐ weil der Anmelder selbst keine Abbildung vorgeschlagen hat.

☐ weil diese Abbildung die Erfindung besser kennzeichnet.

☒ keine der Abb.





10/018483

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- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
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- VIII ☐ Certain observations on the international application

Date of submission of the demand 13/12/2000	Date of completion of this report 12.09.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Grosskopf, R Telephone No. +49 89 2399 8714

**INTERNATIONAL PRELIMINARY  
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International application No. PCT/EP00/05457

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The same applies for the general method of Claim 11 which cannot make any contribution to a possible inventive activity.

(19) World Intellectual Property Organization  
International Bureau



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28 December 2000 (28.12.2000)

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(10) International Publication Number  
**WO 00/78947 A1**

(51) International Patent Classification<sup>7</sup>:  
C07K 14/47, 16/19, 16/18, G01N 33/68

C12N 15/12,

(74) Common Representative: **MERCK PATENT GMBH**;  
Frankfurter Strasse 250, D-64293 Darmstadt (DE).

(21) International Application Number: PCT/EP00/05457

(22) International Filing Date: 14 June 2000 (14.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
99112024.7 22 June 1999 (22.06.1999) EP

(71) Applicant (for all designated States except US): **MERCK PATENT GMBH** [DE/DE]; Frankfurter Strasse 250, D-64293 Darmstadt (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DEN DAAS**, Izaak [DE/DE]; Schillerstrasse 76, D-64407 Fränkisch-Crumbach (DE). **FISCHER**, Viola [DE/DE]; An der Allee 80, D-55122 Mainz (DE). **SEYFRIED**, Christoph [DE/DE]; Mathildenstrasse 6, D-64342 Seeheim (DE). **VON MELCHNER**, Laurie [US/US]; Heinrich Heine-Strasse 6, D-64380 Rossdorf (DE).

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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**Published:**

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HEAD TRAUMA INDUCED CYTOPLASMATIC CALCIUM BINDING PROTEIN

(57) Abstract: ANIC-BP polypeptides and polynucleotides and methods for producing such polypeptides by recombinant techniques are disclosed. Also disclosed are methods for utilizing ANIC-BP polypeptides and polynucleotides in diagnostic assays.



**WO 00/78947 A1**



## Head trauma induced cytoplasmatic calcium binding protein

### Field of the Invention

5 The present invention relates to a human Acute Neural Induced Calcium-Binding Protein and polynucleotides (ANIC-BP) which identify and encode this protein. The invention as well provides expression vectors, host cells and antibodies. In addition the invention provides methods for producing the protein and for treating or preventing disorders associated with the expression of the protein. The invention also relates  
10 to inhibiting or activating the action of such polynucleotides and polypeptides.

### Background of the Invention

15 Stroke and acute head trauma, multiple sclerosis and spinal cord injury are diseases for which so far there is no therapy available. Stroke is the third leading cause of death and the burden for the patients as well as for the social systems are enormous. In case of ischaemic stroke, which accounts for most of the strokes, blood vessel blockade in the brain is an initial event. Head trauma accidents are the leading disease of young people in the western world. A much smaller number of patients is affected  
20 by haemorrhagic stroke caused by mechanical impact and artery rupture. It is a common feature, that approved pharmaceuticals are hardly available. Currently available treatment approaches are based upon pathophysiologic concepts derived from experimental work with focal cerebral ischaemia. These include pharmacological strategies for arterial recanalisation, inhibition of inflammatory processes and neural protection. Further work  
25 with arterial reperfusion strategies is under way. Early clinical studies with polymorphonuclear leukocyte-dependent endothelial adhesion receptor antagonists are being completed, but a strategy has yet to emerge. However any strategy addressing only a single step in the ischaemic cascade is likely to produce a modest benefit only. Therefor future  
30 therapies most properly will be based on combination therapies. A combination of low-dose acetylsalicylic acid (ASA) and modified-release dipyridamole has been shown to be additive in the secondary prevention of stroke (Tijssen et al., Int.J.Clin. Pract., suppl. 91, 14-16, 1997). Another  
35 combination which is currently proposed for clinical evaluation is tPA plus





an effective neuroprotective agent. However the results of these studies are far from being highly effective. Hence, there is urgent need to learn more about the pathophysiological mechanisms in order to provide drug targets that could be used to develop new drugs and establish more generally useful regimens for the treatment of patients suffering from acute neurodamaging effects and disorders such as multiple sclerosis.

This invention does focus on  $\text{Ca}^{2+}$ -binding proteins since there has been considerable evidence for a role of  $\text{Ca}^{2+}$ -binding proteins in neuroprotection. Although the precise function of calcium binding proteins (CaBPs) is not definitively known, it has been proposed that CaBPs act to buffer intracellular  $\text{Ca}^{2+}$  levels. Since  $\text{Ca}^{2+}$  overload activates biochemical processes leading to proteolysis and mitochondrial malfunction, this buffering capacity of CaBPs may have a protective effect against excitotoxic neuronal injury (Heizmann et al., TINS, 15, 259-64, 1992). A variety of evidences exists to support this proposed role of CaBPs in modulation of  $\text{Ca}^{2+}$  levels and in neuroprotection.

The major  $\text{Ca}^{2+}$ -binding proteins (CaBPs) expressed in the central nervous system (parvalbumin, calbindin-D28K, and calretinin) have a very unusual and selective pattern of expression in various neuronal populations. Among neurons that do express CaBPs, most express only one type, although a small number of neurons express more than one of the major calcium binding proteins. There is growing evidence that the presence or absence of CaBPs in particular cell types underlies the phenomenon known as selective vulnerability. Selective vulnerability is a property of specific types of neurons to die in response to particular types of central nervous system (CNS) injury. For example, CA1 hippocampal neurons are selectively vulnerable to global ischemia, cerebellar Purkinje cells are selectively vulnerable to head trauma, stroke, and fetal alcohol exposure, and neurons in the substantia nigra are selectively vulnerable in Parkinson's Disease. An effort has been made to link selective neuronal vulnerability to the expression patterns of various CaBPs and some authors report that high levels of CaBPs are found in neuronal populations that are selectively *vulnerable* to injury, while others report high CaBP levels in neuronal populations that are selectively *resistant* to injury. For example, neurons expressing high levels of parvalbumin are reported to be selectively vulnerable to AMPA-induced toxicity (Weiss et al., Neurol., 40, 1288-1292, 1990), whereas cultured hippocampal



neurons expressing high levels of calbindin-D28K are reported to be selectively resistant to glutamate-induced toxicity (Baimbridge et al., TINS, 15, 303-8, 1992). Similarly, hippocampal neurons expressing high levels of calretinin are resistant to toxic doses of the excitotoxins glutamate, NMDA, kainate, and quisqualate (Winsky et al., in: Novel Calcium-Binding Proteins, 277-300, 1991).

CaBPs have also been shown to have altered expression in various CNS disease states, but again the results are inconsistent about whether CaBP expression is related to selective vulnerability or selective resistance to injury. Neurons expressing calbindin-D28K are reported to be selectively vulnerable in Alzheimer's Disease (Iacopino et al. PNAS, 87, 4078-82, 1990, Hof et al. Exp. Neurol., 111, 293-301, 1991) and Huntington's Disease (Kiyama et al., Brain Res., 526, 303-07, 1990), although calbindin-D28K-expressing neurons in the substantia nigra are not selectively vulnerable in Parkinson's Disease (Yamada et al., Brain Res., 526, 303-07, 1990). In a gerbil model of global ischemia, the presence of parvalbumin in certain hippocampal cell types has been shown to be positively associated with survival (Tortosa et al., Neurosci., 1, 33-43, 1993), although another study suggested that parvalbumin-expressing hippocampal interneurons are selectively vulnerable in Alzheimer's Disease (Brady et al., Neurosci., 80, 1113-25, 1997).

Mice with a knockout of the calbindin gene show functional deficits (e.g. ataxia) that suggest severe dysfunction in neurons normally expressing this CaBP (e.g., cerebellar Purkinje cells) despite the fact that these neurons appear morphologically normal. This finding suggests that CaBPs are vital to cellular activity patterns (Airaksinen et al., PNAS, 94, 1488-93, 1997). In addition, retroviral infection of motoneurons with calbindin-D28k has been shown to have neuroprotective effects against toxicity induced by IgG from patients with amyotrophic lateral sclerosis (Ho et al., PNAS, 93, 6796-801, 1996) and transfection with calbindin-D28k has been shown to protect PC12 cells from toxicity due to serum withdrawal, glutamate exposure, and the neurotoxin MPP+ (McMahon et al., Molec. Brain Res., 526, 303-07, 1998).

In conclusion, there is considerable information regarding a role for calcium binding proteins in neurodegeneration. It is certain that some CaBPs provide protection and others cause selective vulnerability,



however it is not yet clear whether the expression of certain CaBPs within different neuronal populations results in different functional responses of a given CaBP. Another observation is that the severity of different types of CNS injury may affect the apparent neuroprotective efficacy of CaBPs -- i.e., one CaBP may confer resistance in an injury model involving a mild injury, but may be unable to buffer  $\text{Ca}^{2+}$  increases in more severe CNS injuries. Thus there is considerable evidence that CaBPs confer resistance as well as vulnerability in CNS injury process, but the mechanism of this involvement and the regulation of the response has still to be worked out.

A gene family comprising the functionally unidentified gene (MO25) was recently isolated from a mouse derived cDNA library (Miyamoto et al., Mol. Reprod. Dev., 34, 1-7, 1993). The library was constructed from RNA isolated from an early embryonic mouse. The predicted amino acid sequence for Mo25 revealed that the MO25 gene may have structural homology with  $\text{Ca}^{2+}$  binding proteins and lack membrane spanning domains, indicating that the protein might be involved in cytosolic development of the unfertilized egg. However the real function of this protein remains unknown. Another Mo25 like gene, has been cloned from a *Drosophila* cDNA library (Nozaki et al. DNA Cell Biol., 15, 505-09, 1996). The deduced amino acid sequence of the Mo25 cDNA had 69.3% identity with mouse Mo25 homologue. A homologue in *Saccharomyces cerevisiae* encoded in an open reading frame near the calcineurin B subunit gene. Most recently another gene has been isolated from *Aspergillus* hym A mutants (Karos et al., Mol. Gen Genet., 260, 510-521, 1999) and turned out to correspond to the homologues in yeast, plants, fly, worm, fish, mice and man. A cellular function for the Hym protein has not yet been defined in any of the described organisms. As with many other other proteins where the functional contribution is only partially understood the drug discovery process is currently undergoing a fundamental revolution as it embraces "functional genomics", that is, high throughput genome- or gene-based biology. This approach as a means to identify genes and gene products as therapeutic targets is rapidly superceding earlier approaches based on "positional cloning". A phenotype, that is a biological function or genetic disease, would be identified and this would then be tracked back to the responsible gene, based on its genetic map position.



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Functional genomics relies heavily on high-throughput DNA sequencing technologies and the various tools of bioinformatics to identify gene sequences of potential interest from the many molecular biology databases now available. There is a continuing need to identify and characterise further genes and their related polypeptides/proteins, as targets for drug discovery.

### Summary of the Invention

The present invention relates to ANIC-BP, in particular ANIC-BP polypeptides and ANIC-BP polynucleotides, recombinant materials and methods for their production. Such polypeptides and polynucleotides are of interest in relation to methods of treatment of certain diseases, including, but not limited to stroke and acute head trauma, multiple sclerosis and spinal cord injury, hereinafter referred to as "diseases of the invention". In a further aspect, the invention relates to methods for identifying agonists and antagonists (e.g., inhibitors) using the materials provided by the invention, and treating conditions associated with ANIC-BP imbalance with the identified compounds. In a still further aspect, the invention relates to diagnostic assays for detecting diseases associated with inappropriate ANIC-BP activity or levels.

### Description of the Invention

In a first aspect, the present invention relates to ANIC-BP polypeptides. Such polypeptides include:

- (a) an isolated polypeptide encoded by a polynucleotide comprising the sequence of SEQ ID NO:1;
- (b) an isolated polypeptide comprising a polypeptide sequence having at least 95%, 96%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;
- (c) an isolated polypeptide comprising the polypeptide sequence of SEQ ID NO:2;





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(d) an isolated polypeptide having at least 95%, 96%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;

(e) the polypeptide sequence of SEQ ID NO:2; and

5 (f) an isolated polypeptide having or comprising a polypeptide sequence that has an Identity Index of 0.95, 0.96, 0.97, 0.98, or 0.99 compared to the polypeptide sequence of SEQ ID NO:2;

(g) fragments and variants of such polypeptides in (a) to (f).

10 Polypeptides of the present invention are believed to be members of \*Calcium binding protein family of polypeptides. They are therefore of interest because they could serve as a novel drug target.

The biological properties of the ANIC-BP are hereinafter referred to as "biological activity of ANIC-BP " or " ANIC-BP activity". Preferably, a polypeptide of the present invention exhibits at least one biological activity of ANIC-BP.

15 Polypeptides of the present invention also includes variants of the aforementioned polypeptides, including all allelic forms and splice variants. Such polypeptides vary from the reference polypeptide by insertions, deletions, and substitutions that may be conservative or non-conservative, or any combination thereof. Particularly preferred variants are those in  
20 which several, for instance from 50 to 30, from 30 to 20, from 20 to 10, from 10 to 5, from 5 to 3, from 3 to 2, from 2 to 1 or 1 amino acids are inserted, substituted, or deleted, in any combination.

Preferred fragments of polypeptides of the present invention include an isolated polypeptide comprising an amino acid sequence having at least  
25 30, 50 or 100 contiguous amino acids from the amino acid sequence of SEQ ID NO: 2, or an isolated polypeptide comprising an amino acid sequence having at least 30, 50 or 100 contiguous amino acids truncated or deleted from the amino acid sequence of SEQ ID NO: 2. Preferred fragments are biologically active fragments that mediate the biological activity of ANIC-BP, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Also preferred are those  
30 fragments that are antigenic or immunogenic in an animal, especially in a human.



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Fragments of the polypeptides of the invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, these variants may be employed as intermediates for producing the full-length polypeptides of the invention. The polypeptides of the present invention may be in the form of the "mature" protein or may be a part of a larger protein such as a precursor or a fusion protein. It is often advantageous to include an additional amino acid sequence that contains secretory or leader sequences, pro-sequences, sequences that aid in purification, for instance multiple histidine residues, or an additional sequence for stability during recombinant production.

Polypeptides of the present invention can be prepared in any suitable manner, for instance by isolation from naturally occurring sources, from genetically engineered host cells comprising expression systems (*vide infra*) or by chemical synthesis, using for instance automated peptide synthesizers, or a combination of such methods.. Means for preparing such polypeptides are well understood in the art.

In a further aspect, the present invention relates to ANIC-BP polynucleotides. Such polynucleotides include:

- (a) an isolated polynucleotide comprising a polynucleotide sequence having at least 95%, 96%, 97%, 98%, or 99% identity to the polynucleotide sequence of SEQ ID NO:1;
- (b) an isolated polynucleotide comprising the polynucleotide of SEQ ID NO:1;
- (c) an isolated polynucleotide having at least 95%, 96%, 97%, 98%, or 99% identity to the polynucleotide of SEQ ID NO:1;
- (d) the isolated polynucleotide of SEQ ID NO:1;
- (e) an isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide sequence having at least 95%, 96%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;
- (f) an isolated polynucleotide comprising a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2;



- (g) an isolated polynucleotide having a polynucleotide sequence encoding a polypeptide sequence having at least 95%, 96%, 97%, 98%, or 99% identity to the polypeptide sequence of SEQ ID NO:2;
- (h) an isolated polynucleotide encoding the polypeptide of SEQ ID NO:2;
- 5 (i) an isolated polynucleotide having or comprising a polynucleotide sequence that has an Identity Index of 0.95, 0.96, 0.97, 0.98, or 0.99 compared to the polynucleotide sequence of SEQ ID NO:1;
- (j) an isolated polynucleotide having or comprising a polynucleotide sequence encoding a polypeptide sequence that has an Identity Index of 0.95, 0.96, 0.97, 0.98, or 0.99 compared to the polypeptide sequence of SEQ ID NO:2; and polynucleotides that are fragments and variants of the above mentioned polynucleotides or that are complementary to above mentioned polynucleotides, over the entire length thereof.
- 10
- 15 Preferred fragments of polynucleotides of the present invention include an isolated polynucleotide comprising an nucleotide sequence having at least 15, 30, 50 or 100 contiguous nucleotides from the sequence of SEQ ID NO: 1, or an isolated polynucleotide comprising an sequence having at least 30, 50 or 100 contiguous nucleotides truncated or deleted from the sequence of SEQ ID NO: 1.
- 20
- Preferred variants of polynucleotides of the present invention include splice variants, allelic variants, and polymorphisms, including polynucleotides having one or more single nucleotide polymorphisms (SNPs).
- 25
- Polynucleotides of the present invention also include polynucleotides encoding polypeptide variants that comprise the amino acid sequence of SEQ ID NO:2 and in which several, for instance from 50 to 30, from 30 to 20, from 20 to 10, from 10 to 5, from 5 to 3, from 3 to 2, from 2 to 1 or 1 amino acid residues are substituted, deleted or added, in any combination.
- 30
- In a further aspect, the present invention provides polynucleotides that are RNA transcripts of the DNA sequences of the present invention. Accordingly, there is provided an RNA polynucleotide that:



(a) comprises an RNA transcript of the DNA sequence encoding the polypeptide of SEQ ID NO:2;

(b) is the RNA transcript of the DNA sequence encoding the polypeptide of SEQ ID NO:2;

5 (c) comprises an RNA transcript of the DNA sequence of SEQ ID NO:1; or

(d) is the RNA transcript of the DNA sequence of SEQ ID NO:1; and RNA polynucleotides that are complementary thereto.

10 The polynucleotide sequence of SEQ ID NO:1 shows homology with Hym A (Nozaki et al. DNA cell Biol., 15, 505-09, 1996) and Mo25 (Karos et al., Mol. Gen. Genet., 260, 510-521, 1999). The polynucleotide sequence of SEQ ID NO:1 is a cDNA sequence that encodes the polypeptide of SEQ ID NO:2. The polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 may be identical to the polypeptide encoding sequence of SEQ ID NO:1 or it may be a sequence other than SEQ ID NO:1, which, as a result of the redundancy (degeneracy) of the genetic code, also encodes the polypeptide of SEQ ID NO:2. The polypeptide of the SEQ ID NO:2 is related to other proteins of the\*Calcium binding protein family, having homology and/or structural similarity with Hym A and Mo25 proteins.

20 Preferred polypeptides and polynucleotides of the present invention are expected to have, *inter alia*, similar biological functions/properties to their homologous polypeptides and polynucleotides. Furthermore, preferred polypeptides and polynucleotides of the present invention have at least an ANIC-BP activity.

25 Polynucleotides of the present invention may be obtained using standard cloning and screening techniques from a cDNA library derived from mRNA cells of the human central nervous system, (see for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)). Polynucleotides of the invention can also be obtained from natural sources such as genomic DNA libraries or can be synthesized using well known and commercially available techniques.

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When polynucleotides of the present invention are used for the recombinant production of polypeptides of the present invention, the polynucleotide may include the coding sequence for the mature polypeptide, by itself, or the coding sequence for the mature polypeptide in reading frame with other coding sequences, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence, or other fusion peptide portions. For example, a marker sequence that facilitates purification of the fused polypeptide can be encoded. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al.*, Proc Natl Acad Sci USA (1989) 86:821-824, or is an HA tag. The polynucleotide may also contain non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, splicing and polyadenylation signals, ribosome binding sites and sequences that stabilize mRNA.

Polynucleotides that are identical, or have sufficient identity to a polynucleotide sequence of SEQ ID NO:1, may be used as hybridization probes for cDNA and genomic DNA or as primers for a nucleic acid amplification reaction (for instance, PCR). Such probes and primers may be used to isolate full-length cDNAs and genomic clones encoding polypeptides of the present invention and to isolate cDNA and genomic clones of other genes (including genes encoding paralogs from human sources and orthologs and paralogs from species other than humans) that have a high sequence similarity to SEQ ID NO:1, typically at least 95% identity. Preferred probes and primers will generally comprise at least 15 nucleotides, preferably, at least 30 nucleotides and may have at least 50, if not at least 100 nucleotides. Particularly preferred probes will have between 30 and 50 nucleotides. Particularly preferred primers will have between 20 and 25 nucleotides.

A polynucleotide encoding a polypeptide of the present invention, including homologs from species other than humans, may be obtained by a process comprising the steps of screening a library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO: 1 or a fragment thereof, preferably of at least 15 nucleotides; and isolating full-length cDNA and genomic clones containing said polynucleotide sequence. Such hybridization techniques are well known to the skilled artisan. Preferred stringent hybridization conditions include overnight incubation at



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42°C in a solution comprising: 50% formamide, 5xSSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10 % dextran sulfate, and 20 microgram/ml denatured, sheared salmon sperm DNA; followed by washing the filters in 0.1x SSC at about 65°C. Thus the present invention also includes isolated polynucleotides, preferably with a nucleotide sequence of at least 100, obtained by screening a library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO:1 or a fragment thereof, preferably of at least 15 nucleotides.

The skilled artisan will appreciate that, in many cases, an isolated cDNA sequence will be incomplete, in that the region coding for the polypeptide does not extend all the way through to the 5' terminus. This is a consequence of reverse transcriptase, an enzyme with inherently low "processivity" (a measure of the ability of the enzyme to remain attached to the template during the polymerisation reaction), failing to complete a DNA copy of the mRNA template during first strand cDNA synthesis.

There are several methods available and well known to those skilled in the art to obtain full-length cDNAs, or extend short cDNAs, for example those based on the method of Rapid Amplification of cDNA ends (RACE) (see, for example, Frohman et al., Proc Nat Acad Sci USA 85, 8998-9002, 1988). Recent modifications of the technique, exemplified by the Marathon (trade mark) technology (Clontech Laboratories Inc.) for example, have significantly simplified the search for longer cDNAs. In the Marathon (trade mark) technology, cDNAs have been prepared from mRNA extracted from a chosen tissue and an 'adaptor' sequence ligated onto each end. Nucleic acid amplification (PCR) is then carried out to amplify the "missing" 5' end of the cDNA using a combination of gene specific and adaptor specific oligonucleotide primers. The PCR reaction is then repeated using 'nested' primers, that is, primers designed to anneal within the amplified product (typically an adaptor specific primer that anneals further 3' in the adaptor sequence and a gene specific primer that anneals further 5' in the known gene sequence). The products of this reaction can then be analysed by DNA sequencing and a full-length cDNA constructed either by joining the product directly to the existing cDNA to give a complete sequence, or carrying out a separate full-length PCR using the new sequence information for the design of the 5' primer.



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Recombinant polypeptides of the present invention may be prepared by processes well known in the art from genetically engineered host cells comprising expression systems. Accordingly, in a further aspect, the present invention relates to expression systems comprising a polynucleotide or polynucleotides of the present invention, to host cells which are genetically engineered with such expression systems and to the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof for polynucleotides of the present invention. Polynucleotides may be introduced into host cells by methods described in many standard laboratory manuals, such as Davis et al., Basic Methods in Molecular Biology (1986) and Sambrook et al. (*ibid*). Preferred methods of introducing polynucleotides into host cells include, for instance, calcium phosphate transfection, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

Representative examples of appropriate hosts include bacterial cells, such as *Streptococci*, *Staphylococci*, *E. coli*, *Streptomyces* and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, HEK 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used, for instance, chromosomal, episomal and virus-derived systems, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression systems may contain control regions that regulate as well as engender expression. Generally, any



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system or vector that is able to maintain, propagate or express a polynucleotide to produce a polypeptide in a host may be used. The appropriate polynucleotide sequence may be inserted into an expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, (*ibid*). Appropriate secretion signals may be incorporated into the desired polypeptide to allow secretion of the translated protein into the lumen of the endoplasmic reticulum, the periplasmic space or the extracellular environment. These signals may be endogenous to the polypeptide or they may be heterologous signals.

If a polypeptide of the present invention is to be expressed for use in screening assays, it is generally preferred that the polypeptide be produced at the surface of the cell. In this event, the cells may be harvested prior to use in the screening assay. If the polypeptide is secreted into the medium, the medium can be recovered in order to recover and purify the polypeptide. If produced intracellularly, the cells must first be lysed before the polypeptide is recovered.

Polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding proteins may be employed to regenerate active conformation when the polypeptide is denatured during intracellular synthesis, isolation and/or purification.

Polynucleotides of the present invention may be used as diagnostic reagents, through detecting mutations in the associated gene. Detection of a mutated form of the gene characterised by the polynucleotide of SEQ ID NO:1 in the cDNA or genomic sequence and which is associated with a dysfunction will provide a diagnostic tool that can add to, or define, a diagnosis of a disease, or susceptibility to a disease, which results from under-expression, over-expression or altered spatial or temporal expression of the gene. Individuals carrying mutations in the gene may be detected at the DNA level by a variety of techniques well known in the art.





Nucleic acids for diagnosis may be obtained from a subject's cells, such as from blood, urine, saliva, tissue biopsy or autopsy material. The genomic DNA may be used directly for detection or it may be amplified enzymatically by using PCR, preferably RT-PCR, or other amplification techniques prior to analysis. RNA or cDNA may also be used in similar fashion. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to labeled ANIC-BP nucleotide sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence difference may also be detected by alterations in the electrophoretic mobility of DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing (see, for instance, Myers *et al.*, Science (1985) 230:1242). Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (see Cotton *et al.*, Proc Natl Acad Sci USA (1985) 85: 4397-4401).

An array of oligonucleotides probes comprising ANIC-BP polynucleotide sequence or fragments thereof can be constructed to conduct efficient screening of *e.g.*, genetic mutations. Such arrays are preferably high density arrays or grids. Array technology methods are well known and have general applicability and can be used to address a variety of questions in molecular genetics including gene expression, genetic linkage, and genetic variability, see, for example, M.Chee *et al.*, Science, 274, 610-613 (1996) and other references cited therein.

Detection of abnormally decreased or increased levels of polypeptide or mRNA expression may also be used for diagnosing or determining susceptibility of a subject to a disease of the invention. Decreased or increased expression can be measured at the RNA level using any of the methods well known in the art for the quantitation of polynucleotides, such as, for example, nucleic acid amplification, for instance PCR, RT-PCR, RNase protection, Northern blotting and other hybridization methods. Assay techniques that can be used to determine levels of a protein, such as a polypeptide of the present invention, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.



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Thus in another aspect, the present invention relates to a diagnostic kit comprising:

(a) a polynucleotide of the present invention, preferably the nucleotide sequence of SEQ ID NO: 1, or a fragment or an RNA transcript thereof;

5 (b) a nucleotide sequence complementary to that of (a);

(c) a polypeptide of the present invention, preferably the polypeptide of SEQ ID NO:2 or a fragment thereof; or

(d) an antibody to a polypeptide of the present invention, preferably to the polypeptide of SEQ ID NO:2.

10 It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component. Such a kit will be of use in diagnosing a disease or susceptibility to a disease, particularly diseases of the invention, amongst others.

15 The polynucleotide sequences of the present invention are valuable for chromosome localisation studies. The sequence is specifically targeted to, and can hybridize with, a particular location on an individual human chromosome. The mapping of relevant sequences to chromosomes according to the present invention is an important first step in correlating those sequences with gene associated disease. Once a sequence has  
20 been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found in, for example, V. McKusick, Mendelian Inheritance in Man (available on-line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been  
25 mapped to the same chromosomal region are then identified through linkage analysis (co-inheritance of physically adjacent genes). Precise human chromosomal localisations for a genomic sequence (gene fragment etc.) can be determined using Radiation Hybrid (RH) Mapping (Walter, M. Spillett, D., Thomas, P., Weissenbach, J., and Goodfellow, P.,  
30 (1994) A method for constructing radiation hybrid maps of whole genomes, Nature Genetics 7, 22-28). A number of RH panels are available from Research Genetics (Huntsville, AL, USA) e.g. the GeneBridge4 RH panel (Hum Mol Genet 1996 Mar;5(3):339-46 A radiation hybrid map of the human genome. Gyapay G, Schmitt K,



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Fizames C, Jones H, Vega-Czarny N, Spillett D, Muselet D, Prud'Homme JF, Dib C, Auffray C, Morissette J, Weissenbach J, Goodfellow PN). To determine the chromosomal location of a gene using this panel, 93 PCRs are performed using primers designed from the gene of interest on RH DNAs. Each of these DNAs contains random human genomic fragments maintained in a hamster background (human / hamster hybrid cell lines). These PCRs result in 93 scores indicating the presence or absence of the PCR product of the gene of interest. These scores are compared with scores created using PCR products from genomic sequences of known location. This comparison is conducted at <http://www.genome.wi.mit.edu>

The polynucleotide sequences of the present invention are also valuable tools for tissue expression studies. Such studies allow the determination of expression patterns of polynucleotides of the present invention which may give an indication as to the expression patterns of the encoded polypeptides in tissues, by detecting the mRNAs that encode them. The techniques used are well known in the art and include in situ hybridisation techniques to clones arrayed on a grid, such as cDNA microarray hybridisation (Schena *et al*, Science, 270, 467-470, 1995 and Shalon *et al*, Genome Res, 6, 639-645, 1996) and nucleotide amplification techniques such as PCR. A preferred method uses the TAQMAN (Trade mark) technology available from Perkin Elmer. Results from these studies can provide an indication of the normal function of the polypeptide in the organism. In addition, comparative studies of the normal expression pattern of mRNAs with that of mRNAs encoded by an alternative form of the same gene (for example, one having an alteration in polypeptide coding potential or a regulatory mutation) can provide valuable insights into the role of the polypeptides of the present invention, or that of inappropriate expression thereof in disease. Such inappropriate expression may be of a temporal, spatial or simply quantitative nature. The polypeptides of the present invention are expressed in human brain.

A further aspect of the present invention relates to antibodies. The polypeptides of the invention or their fragments, or cells expressing them, can be used as immunogens to produce antibodies that are immunospecific for polypeptides of the present invention. The term "immunospecific" means that the antibodies have substantially greater affinity for the



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polypeptides of the invention than their affinity for other related polypeptides in the prior art.

Antibodies generated against polypeptides of the present invention may be obtained by administering the polypeptides or epitope-bearing fragments, or cells to an animal, preferably a non-human animal, using routine protocols. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., Nature (1975) 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, Immunology Today (1983) 4:72) and the EBV-hybridoma technique (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, 77-96, Alan R. Liss, Inc., 1985).

Techniques for the production of single chain antibodies, such as those described in U.S. Patent No. 4,946,778, can also be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms, including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography. Antibodies against polypeptides of the present invention may also be employed to treat diseases of the invention, amongst others.

Polypeptides and polynucleotides of the present invention may also be used as vaccines. Accordingly, in a further aspect, the present invention relates to a method for inducing an immunological response in a mammal that comprises inoculating the mammal with a polypeptide of the present invention, adequate to produce antibody and/or T cell immune response, including, for example, cytokine-producing T cells or cytotoxic T cells, to protect said animal from disease, whether that disease is already established within the individual or not. An immunological response in a mammal may also be induced by a method comprises delivering a polypeptide of the present invention *via* a vector directing expression of the polynucleotide and coding for the polypeptide *in vivo* in order to induce such an immunological response to produce antibody to protect said animal from diseases of the invention. One way of administering the vector is by accelerating it into the desired cells as a coating on particles





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or otherwise. Such nucleic acid vector may comprise DNA, RNA, a modified nucleic acid, or a DNA/RNA hybrid. For use as a vaccine, a polypeptide or a nucleic acid vector will be normally provided as a vaccine formulation (composition). The formulation may further comprise a suitable carrier. Since a polypeptide may be broken down in the stomach, it is preferably administered parenterally (for instance, subcutaneous, intramuscular, intravenous, or intradermal injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions that may contain anti-oxidants, buffers, bacteriostats and solutes that render the formulation isotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions that may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

Polypeptides of the present invention have one or more biological functions that are of relevance in one or more disease states, in particular the diseases of the invention hereinbefore mentioned. It is therefore useful to identify compounds that stimulate or inhibit the function or level of the polypeptide. Accordingly, in a further aspect, the present invention provides for a method of screening compounds to identify those that stimulate or inhibit the function or level of the polypeptide. Such methods identify agonists or antagonists that may be employed for therapeutic and prophylactic purposes for such diseases of the invention as hereinbefore mentioned. Compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, collections of chemical compounds, and natural product mixtures. Such agonists or antagonists so-identified may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of the polypeptide; a structural or functional mimetic thereof (see Coligan *et al.*, Current Protocols in Immunology 1(2):Chapter 5 (1991)) or a small molecule.



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The screening method may simply measure the binding of a candidate compound to the polypeptide, or to cells or membranes bearing the polypeptide, or a fusion protein thereof, by means of a label directly or indirectly associated with the candidate compound. Alternatively, the screening method may involve measuring or detecting (qualitatively or quantitatively) the competitive binding of a candidate compound to the polypeptide against a labeled competitor (e.g. agonist or antagonist). Further, these screening methods may test whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide, using detection systems appropriate to the cells bearing the polypeptide. Inhibitors of activation are generally assayed in the presence of a known agonist and the effect on activation by the agonist by the presence of the candidate compound is observed. Further, the screening methods may simply comprise the steps of mixing a candidate compound with a solution containing a polypeptide of the present invention, to form a mixture, measuring a ANIC-BP activity in the mixture, and comparing the ANIC-BP activity of the mixture to a control mixture which contains no candidate compound.

Polypeptides of the present invention may be employed in conventional low capacity screening methods and also in high-throughput screening (HTS) formats. Such HTS formats include not only the well-established use of 96- and, more recently, 384-well micotiter plates but also emerging methods such as the nanowell method described by Schullek et al, Anal Biochem., 246, 20-29, (1997).

Fusion proteins, such as those made from Fc portion and ANIC-BP polypeptide, as hereinbefore described, can also be used for high-throughput screening assays to identify antagonists for the polypeptide of the present invention (see D. Bennett *et al.*, J Mol Recognition, 8:52-58 (1995); and K. Johanson *et al.*, J Biol Chem, 270(16):9459-9471 (1995)).



### Screening techniques

The polynucleotides, polypeptides and antibodies to the polypeptide of the present invention may also be used to configure screening methods for detecting the effect of added compounds on the production of mRNA and polypeptide in cells. For example, an ELISA assay may be constructed for measuring secreted or cell associated levels of polypeptide using monoclonal and polyclonal antibodies by standard methods known in the art. This can be used to discover agents that may inhibit or enhance the production of polypeptide (also called antagonist or agonist, respectively) from suitably manipulated cells or tissues.

A polypeptide of the present invention may be used to identify membrane bound or soluble receptors, if any, through standard receptor binding techniques known in the art. These include, but are not limited to, ligand binding and crosslinking assays in which the polypeptide is labeled with a radioactive isotope (for instance,  $^{125}\text{I}$ ), chemically modified (for instance, biotinylated), or fused to a peptide sequence suitable for detection or purification, and incubated with a source of the putative receptor (cells, cell membranes, cell supernatants, tissue extracts, bodily fluids). Other methods include biophysical techniques such as surface plasmon resonance and spectroscopy. These screening methods may also be used to identify agonists and antagonists of the polypeptide that compete with the binding of the polypeptide to its receptors, if any. Standard methods for conducting such assays are well understood in the art.

Examples of antagonists of polypeptides of the present invention include antibodies or, in some cases, oligonucleotides or proteins that are closely related to the ligands, substrates, receptors, enzymes, etc., as the case may be, of the polypeptide, e.g., a fragment of the ligands, substrates, receptors, enzymes, etc.; or a small molecule that bind to the polypeptide of the present invention but do not elicit a response, so that the activity of the polypeptide is prevented.

Screening methods may also involve the use of transgenic technology and ANIC-BP gene. The art of constructing transgenic animals is well established. For example, the ANIC-BP gene may be introduced through microinjection into the male pronucleus of fertilized oocytes, retroviral transfer into pre- or post-implantation embryos, or injection of genetically



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modified, such as by electroporation, embryonic stem cells into host blastocysts. Particularly useful transgenic animals are so-called "knock-in" animals in which an animal gene is replaced by the human equivalent within the genome of that animal. Knock-in transgenic animals are useful in the drug discovery process, for target validation, where the compound is specific for the human target. Other useful transgenic animals are so-called "knock-out" animals in which the expression of the animal ortholog of a polypeptide of the present invention and encoded by an endogenous DNA sequence in a cell is partially or completely annulled. The gene knock-out may be targeted to specific cells or tissues, may occur only in certain cells or tissues as a consequence of the limitations of the technology, or may occur in all, or substantially all, cells in the animal. Transgenic animal technology also offers a whole animal expression-cloning system in which introduced genes are expressed to give large amounts of polypeptides of the present invention

Screening kits for use in the above described methods form a further aspect of the present invention. Such screening kits comprise:

- (a) a polypeptide of the present invention;
- (b) a recombinant cell expressing a polypeptide of the present invention;
- (c) a cell membrane expressing a polypeptide of the present invention; or
- (d) an antibody to a polypeptide of the present invention;

which polypeptide is preferably that of SEQ ID NO:2.

It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component.

### Glossary

The following definitions are provided to facilitate understanding of certain terms used frequently hereinbefore.

"Antibodies" as used herein includes polyclonal and monoclonal antibodies, chimeric, single chain, and humanized antibodies, as well as





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Fab fragments, including the products of an Fab or another immunoglobulin expression library.

"Isolated" means altered "by the hand of man" from its natural state, *i.e.*, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Moreover, a polynucleotide or polypeptide that is introduced into an organism by transformation, genetic manipulation or by any other recombinant method is "isolated" even if it is still present in said organism, which organism may be living or non-living.

"Polynucleotide" generally refers to any polyribonucleotide (RNA) or polydeoxiribonucleotide (DNA), which may be unmodified or modified RNA or DNA. "Polynucleotides" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term "polynucleotide" also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications may be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

"Polypeptide" refers to any polypeptide comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, *i.e.*, peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may



contain amino acids other than the 20 gene-encoded amino acids. "Polypeptides" include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques that are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications may occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present to the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, biotinylation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination (see, for instance, *Proteins - Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., *Post-translational Protein Modifications: Perspectives and Prospects*, 1-12, in *Post-translational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, "Analysis for protein modifications and nonprotein cofactors", *Meth Enzymol*, 182, 626-646, 1990, and Rattan *et al.*, "Protein Synthesis: Post-translational Modifications and Aging", *Ann NY Acad Sci*, 663, 48-62, 1992).

"Fragment" of a polypeptide sequence refers to a polypeptide sequence that is shorter than the reference sequence but that retains essentially the same biological function or activity as the reference polypeptide.



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"Fragment" of a polynucleotide sequence refers to a polynucleotide sequence that is shorter than the reference sequence of SEQ ID NO:1..

5 "Variant" refers to a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide, but retains the essential properties thereof. A typical variant of a polynucleotide differs in nucleotide sequence from the reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from the reference polypeptide. Generally, alterations are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, insertions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. Typical conservative substitutions include Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe and Tyr. A variant of a polynucleotide or polypeptide may be naturally occurring such as an allele, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques or by direct synthesis. Also included as variants are polypeptides having one or more post-translational modifications, for instance glycosylation, phosphorylation, methylation, ADP ribosylation and the like. Embodiments include methylation of the N-terminal amino acid, phosphorylations of serines and threonines and modification of C-terminal glycines.

30 "Allele" refers to one of two or more alternative forms of a gene occurring at a given locus in the genome.

"Polymorphism" refers to a variation in nucleotide sequence (and encoded polypeptide sequence, if relevant) at a given position in the genome within a population.



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"Single Nucleotide Polymorphism" (SNP) refers to the occurrence of nucleotide variability at a single nucleotide position in the genome, within a population. An SNP may occur within a gene or within intergenic regions of the genome. SNPs can be assayed using Allele Specific Amplification (ASA). For the process at least 3 primers are required. A common primer is used in reverse complement to the polymorphism being assayed. This common primer can be between 50 and 1500 bps from the polymorphic base. The other two (or more) primers are identical to each other except that the final 3' base wobbles to match one of the two (or more) alleles that make up the polymorphism. Two (or more) PCR reactions are then conducted on sample DNA, each using the common primer and one of the Allele Specific Primers.

"Splice Variant" as used herein refers to cDNA molecules produced from RNA molecules initially transcribed from the same genomic DNA sequence but which have undergone alternative RNA splicing. Alternative RNA splicing occurs when a primary RNA transcript undergoes splicing, generally for the removal of introns, which results in the production of more than one mRNA molecule each of that may encode different amino acid sequences. The term splice variant also refers to the proteins encoded by the above cDNA molecules.

"Identity" reflects a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, determined by comparing the sequences. In general, identity refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of the two polynucleotide or two polypeptide sequences, respectively, over the length of the sequences being compared.

"% Identity" - For sequences where there is not an exact correspondence, a "% identity" may be determined. In general, the two sequences to be compared are aligned to give a maximum correlation between the sequences. This may include inserting "gaps" in either one or both sequences, to enhance the degree of alignment. A % identity may be determined over the whole length of each of the sequences being compared (so-called global alignment), that is particularly suitable for sequences of the same or very similar length, or over shorter, defined lengths (so-called local alignment), that is more suitable for sequences of unequal length.





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"Similarity" is a further, more sophisticated measure of the relationship between two polypeptide sequences. In general, "similarity" means a comparison between the amino acids of two polypeptide chains, on a residue by residue basis, taking into account not only exact correspondences between a between pairs of residues, one from each of the sequences being compared (as for identity) but also, where there is not an exact correspondence, whether, on an evolutionary basis, one residue is a likely substitute for the other. This likelihood has an associated "score" from which the "% similarity" of the two sequences can then be determined.

Methods for comparing the identity and similarity of two or more sequences are well known in the art. Thus for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux J et al, Nucleic Acids Res, 12, 387-395, 1984, available from Genetics Computer Group, Madison, Wisconsin, USA), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides and the % identity and the % similarity between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman (J Mol Biol, 147,195-197, 1981, Advances in Applied Mathematics, 2, 482-489, 1981) and finds the best single region of similarity between two sequences. BESTFIT is more suited to comparing two polynucleotide or two polypeptide sequences that are dissimilar in length, the program assuming that the shorter sequence represents a portion of the longer. In comparison, GAP aligns two sequences, finding a "maximum similarity", according to the algorithm of Neddleman and Wunsch (J Mol Biol, 48, 443-453, 1970). GAP is more suited to comparing sequences that are approximately the same length and an alignment is expected over the entire length. Preferably, the parameters "Gap Weight" and "Length Weight" used in each program are 50 and 3, for polynucleotide sequences and 12 and 4 for polypeptide sequences, respectively. Preferably, % identities and similarities are determined when the two sequences being compared are optimally aligned.

Other programs for determining identity and/or similarity between sequences are also known in the art, for instance the BLAST family of programs (Altschul S F et al, J Mol Biol, 215, 403-410, 1990, Altschul S F et al, Nucleic Acids Res., 25:389-3402, 1997, available from the National



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Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA and accessible through the home page of the NCBI at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and FASTA (Pearson W R, Methods in Enzymology, 183, 63-99, 1990; Pearson W R and Lipman D J, Proc Nat Acad Sci USA, 85, 2444-2448, 1988, available as part of the Wisconsin Sequence Analysis Package).

Preferably, the BLOSUM62 amino acid substitution matrix (Henikoff S and Henikoff J G, Proc. Nat. Acad Sci. USA, 89, 10915-10919, 1992) is used in polypeptide sequence comparisons including where nucleotide sequences are first translated into amino acid sequences before comparison.

Preferably, the program BESTFIT is used to determine the % identity of a query polynucleotide or a polypeptide sequence with respect to a reference polynucleotide or a polypeptide sequence, the query and the reference sequence being optimally aligned and the parameters of the program set at the default value, as hereinbefore described.

"Identity Index" is a measure of sequence relatedness which may be used to compare a candidate sequence (polynucleotide or polypeptide) and a reference sequence. Thus, for instance, a candidate polynucleotide sequence having, for example, an Identity Index of 0.95 compared to a reference polynucleotide sequence is identical to the reference sequence except that the candidate polynucleotide sequence may include on average up to five differences per each 100 nucleotides of the reference sequence. Such differences are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion. These differences may occur at the 5' or 3' terminal positions of the reference polynucleotide sequence or anywhere between these terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. In other words, to obtain a polynucleotide sequence having an Identity Index of 0.95 compared to a reference polynucleotide sequence, an average of up to 5 in every 100 of the nucleotides of the in the reference sequence may be deleted, substituted or inserted, or any combination thereof, as hereinbefore described. The same applies *mutatis mutandis* for other values of the Identity Index, for instance 0.96, 0.97, 0.98 and 0.99.



Similarly, for a polypeptide, a candidate polypeptide sequence having, for example, an Identity Index of 0.95 compared to a reference polypeptide sequence is identical to the reference sequence except that the polypeptide sequence may include an average of up to five differences per each 100 amino acids of the reference sequence. Such differences are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion. These differences may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between these terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. In other words, to obtain a polypeptide sequence having an Identity Index of 0.95 compared to a reference polypeptide sequence, an average of up to 5 in every 100 of the amino acids in the reference sequence may be deleted, substituted or inserted, or any combination thereof, as hereinbefore described. The same applies *mutatis mutandis* for other values of the Identity Index, for instance 0.96, 0.97, 0.98 and 0.99.

The relationship between the number of nucleotide or amino acid differences and the Identity Index may be expressed in the following equation:

$$n_a \leq x_a - (x_a \bullet I),$$

in which:

$n_a$  is the number of nucleotide or amino acid differences,

$x_a$  is the total number of nucleotides or amino acids in SEQ ID NO:1 or SEQ ID NO:2, respectively,

$I$  is the Identity Index ,

$\bullet$  is the symbol for the multiplication operator, and

in which any non-integer product of  $x_a$  and  $I$  is rounded down to the nearest integer prior to subtracting it from  $x_a$ .

"Homolog" is a generic term used in the art to indicate a polynucleotide or polypeptide sequence possessing a high degree of



sequence relatedness to a reference sequence. Such relatedness may be quantified by determining the degree of identity and/or similarity between the two sequences as hereinbefore defined. Falling within this generic term are the terms "ortholog", and "paralog". "Ortholog" refers to a polynucleotide or polypeptide that is the functional equivalent of the polynucleotide or polypeptide in another species. "Paralog" refers to a polynucleotide or polypeptide that within the same species which is functionally similar.

"Fusion protein" refers to a protein encoded by two, unrelated, fused genes or fragments thereof. Examples have been disclosed in US 5541087, US 5726044, EP 574395, EP 493418 and EP 0232 262. In the case of Fc-ANIC-BP, employing an immunoglobulin Fc region as a part of a fusion protein is advantageous for performing the functional expression of Fc-ANIC-BP, to improve pharmacokinetic properties of such a fusion protein when used for therapy and to generate a dimeric Fc-ANIC-BP. The Fc-ANIC-BP DNA construct comprises in 5' to 3' direction, a secretion cassette, i.e. a signal sequence that triggers export from a mammalian cell, DNA encoding an immunoglobulin Fc region fragment, as a fusion partner, and a DNA encoding Fc-ANIC-BP. In some uses it would be desirable to be able to alter the intrinsic functional properties (complement binding, Fc-Receptor binding) by mutating the functional Fc sides while leaving the rest of the fusion protein untouched or delete the Fc part completely after expression.

All publications and references, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference in their entirety as if each individual publication or reference were specifically and individually indicated to be incorporated by reference herein as being fully set forth. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety in the manner described above for publications and references.





**Figure Legends:****Figure 1**

5 Representative mRNA differential display gel with differentially regulated band in the cerebellar hemisphere contralateral to the side of injury. The arrow marks the differentially expressed band.

**Figure 2**

10 RT-PCR with the 270 bp gene fragment of rat Mo25 in the cerebellum after TBI at cycles 5,10,15,20,25 and 30, respectively. Notice the strong expression in the L2 lane at the cycles 20, 25 and 30.

L2: cDNA of TBI rat non lesioned side; R2: cDNA of TBI rat lesioned side.

**Figure 3**

15 Dot blot analysis with radioactive  $^{32}\text{P}$  marked 270 bp gene fragment of rat Mo25 with cerebellum mRNA of rat after TBI. Six individual rats were examined, 3 TBI treated and 3 sham operated animals.

**Figure 4**

20 Multiple tissue Northern blots (Clontech Laboratories Inc, Palo Alto, CA, USA), with radioactive  $^{32}\text{P}$  marked 270 bp gene fragment of rat Mo25 hybridised on 8 rat tissues



### Figure 5

In situ hybridisation of rat brain sections. Sense and antisense probes specific for SICCBP and Mo25 have been used. Strong signals lighting up myelinated nerve tracts (Corpus Callosum, Tractus opticus, tractus olfactorius intermedius, cerebellum commissura anterior) have been monitored using the antisense probes.

### Figure 6

Real Time TaqMan PCR expression of ANIC-BP in rat 7 days after traumatic brain injury. Non lesioned cortical side, lesioned cortical side and cerebellum were compared from sham operated rat brain and brain tissue from rats sacrificed 7 days after the experiment. Error bars are standard error of the mean (S.E.M.).

## Further Examples

### Example 1

#### Traumatic brain injury model

The identification of genes up or down regulated in response to traumatic brain injury (TBI), was studied in rats using the lateral fluid method. A moderate TBI centered on the right parietal cortex was induced with the lateral fluid percussion method in male Sprague-Dawley rats. Five days after TBI, rats were sacrificed and dissected brain tissue was analyzed by differential display. Upregulation of protein in cerebellum of traumatized brains was confirmed by RT-PCR.

Control rats were anesthetized and the temporal muscle was retracted, but no craniotomy was performed. After a five-day survival, all rats were anesthetized, killed, and the brain was dissected and frozen in liquid nitrogen.

A second series of TBI rats were used for histochemical and immunohistochemical staining. One week after TBI, these rats were



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anesthetized and perfused with saline followed by 3% paraformaldehyde. The brains were cut on a freezing microtome into 30 µm coronal sections.

## Example 2

### 5 Immunohistochemistry

Cerebellar sections were labeled with monoclonal antibodies against the calcium binding protein. The immuno complex was visualized using the avidin-biotin/DAB method. As a positive control the Calbindin-D (28kD) has been used as a reliable marker of cerebellar Purkinje cells.

10

## Example 3

### In situ hybridisation

Oligodeoxynucleotides selected from Sequence No.1 (sence, antisence) have been designed according to standard methods apperent to those skilled in the art. These probes have been used for in situ hybridisation in  
15 rat brain tissue sections according to methods known in the art. Autoradiograph have been visualized after 5 day exposure on Kodak BioMax-Film.

### 20 Example 4

#### RNA-Isolation from TBI rats

mRNA differential display has been developed as a method to identify and analyze altered gene expression at the mRNA level in any eukaryotic cell (Liang and Pardee, Science 257, 967,1992). In this invention we  
25 used this method to study genes up or down regulated in response to traumatic brain injury, in order to obtain a better insight into the molecular effects of CNS injury (den Daas et al.; Meeting of the American Neuroscience Society Washington D.C., USA; 1998). The animal model for traumatic brain injury is called the lateral fluid percussion model and is  
30 performed as follows: a moderate traumatic brain injury centered on the



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right parietal cortex of the brain was induced with the lateral fluid percussion method in male Sprague-Dawley rats. Control rats were anesthetized and the temporal muscle was retracted, but no craniotomy was performed. After a five-day survival, rats were anesthetized, killed, the brain was dissected and frozen in liquid nitrogen. Whole brains were homogenized and total RNA was isolated (Sambrook et al. 1989).

### Differential Display

Obtained RNA was analyzed by RNA differential display. Reverse transcription of mRNAs was performed using oligo-dT primers with two additional nucleotides in all possible combinations (downstream primers; 13 mere), thus anchoring the reaction to the beginning of the poly (A) tail. Amplification of the cDNA was conducted with the same 3' primer and a second decamer arbitrary 5' primer (upstream primer). Amplification products were analyzed on a nondenaturing 10% polyacrylamide gel (Amersham Pharmacia Biotech, Germany). DNA was visualized by silver staining. After staining, gels were dried for one hour at room temperature (Figure 1). Differentially regulated bands were cut out from the gel. DNA was eluted, reamplified and subcloned into pCR2.1 vector (Invitrogen, USA). Subcloned fragments were sequenced by the Sanger method (Sanger F., et al, PNAS, USA 74, 5463-5467) and sequences were compared to genomic databases. Validation of the obtained gene fragment was done with reverse transcription PCR (RT-PCR). For confirmation of the differentially expressed rat Mo25, sequence was analyzed by RT-PCR using the Titan one tube RT-PCR system (Boehringer Mannheim, Germany) with specific 19 mere and 21 mere primers. One µg of total RNA from control and TBI animals were used for the RT-PCR. More gene validation has been performed using the dotblot technique with probes from rat Mo25 and Northern blot analysis performed with probes from human Mo25.

### Reverse Transcription PCR (RT-PCR)

For confirmation of the differentially expressed stroke induced calcium binding protein, sequence was analyzed by RT-PCR using the Titan One





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Tube RT-PCR system (Boehringer Mannheim, Germany) with specific 19 mere and 21 mere primers. One  $\mu$ g of total RNA from control and TBI animals were used for the RT-PCR.

5      **Real Time PCR**

Distribution of ANIC-BP in the rat brain after head trauma was examined by the Real Time TaqMan PCR technique in a ABI prism 7700 sequence detection system (PE, Applied Biosystems, Germany). With this technique, absolute concentrations of mRNA can be measured with high  
10      sensitivity. Special primers with a length of 25 and 29 bp and a 32 mere TaqMan probe (reporter dye: FAM/ quencher dye: TAMRA) were designed.

**Ca<sup>2+</sup> binding**

15      Protein separated by SDS-PAGE was blotted onto nitro cellulose membrane and assayed for binding of radiolabled Ca <sup>2+</sup> using the method described by Maruyama, K. et al. (J. Biochem. 95:511-519, 1984). After incubation the surplus of isotope was washed off and the membrane was exposed to Kodak XOMAT.TM film for an appropriate  
20      time. A band developed at the position of the binding protein. The presence of the binding protein was confirmed by using antibody specific for the binding protein and applying that antibody by the western blot method that is well known in the art.



## SEQUENCE LISTING

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    Thr Arg His Lys Leu Leu Ser Ala Glu Phe Leu Glu Gln His Tyr Asp
    195      200      205
    Arg Phe Phe Ser Glu Tyr Glu Lys Leu Leu His Ser Glu Asn Tyr Val
35 210      215      220
    Thr Lys Arg Gln Ser Leu Lys Leu Leu Gly Glu Leu Leu Leu Asp Arg
    225      230      235      240
    His Asn Phe Thr Ile Met Thr Lys Tyr Ile Ser Lys Pro Glu Asn Leu
        245      250      255
40 Lys Leu Met Met Asn Leu Leu Arg Asp Lys Ser Arg Asn Ile Gln Phe
    260      265      270
    Glu Ala Phe His Val Phe Lys Val Phe Val Ala Asn Pro Asn Lys Thr
    275      280      285
    Gln Pro Ile Leu Asp Ile Leu Leu Lys Asn Gln Ala Lys Leu Ile Glu
45 290      295      300
    Phe Leu Ser Lys Phe Gln Asn Asp Arg Thr Glu Asp Glu Gln Phe Asn
    305      310      315      320
    Asp Glu Lys Thr Tyr Leu Val Lys Gln Ile Arg Asp Leu Lys Arg Pro
        325      330      335
50 Ala Gln Gln Glu Ala
    340

```





## Claims

1. An isolated polypeptide selected from one of the groups consisting of:
  - (a) an isolated polypeptide encoded by a polynucleotide comprising the sequence of SEQ ID NO:1;
  - 5 (b) an isolated polypeptide comprising a polypeptide sequence having at least 95% identity to the polypeptide sequence of SEQ ID NO:2;
  - c) an isolated polypeptide having at least 95% identity to the polypeptide sequence of SEQ ID NO:2; and
  - d) the polypeptide sequence of SEQ ID NO:2 and
  - 10 (e) fragments and variants of such polypeptides in (a) to (d).
2. The isolated polypeptide as claimed in claim 1 comprising the polypeptide sequence of SEQ ID NO:2.
- 15 3. The isolated polypeptide as claimed in claim 1 which is the polypeptide sequence of SEQ ID NO:2.
4. An isolated polynucleotide selected from one of the groups consisting of:
  - (a) an isolated polynucleotide comprising a polynucleotide sequence having at least 95% identity to the polynucleotide sequence of SEQ ID NO:1;
  - 20 (b) an isolated polynucleotide having at least 95% identity to the polynucleotide of SEQ ID NO:1;
  - (c) an isolated polynucleotide comprising a polynucleotide sequence encoding a polypeptide sequence having at least 95% identity to the polypeptide sequence of SEQ ID NO:2;
  - 25 (d) an isolated polynucleotide having a polynucleotide sequence encoding a polypeptide sequence having at least 95% identity to the polypeptide sequence of SEQ ID NO:2;



- (e) an isolated polynucleotide with a nucleotide sequence of at least 100 nucleotides obtained by screening a library under stringent hybridization conditions with a labeled probe having the sequence of SEQ ID NO: 1 or a fragment thereof having at least 15 nucleotides;
- 5 (f) a polynucleotide which is the RNA equivalent of a polynucleotide of (a) to (e);
- or a polynucleotide sequence complementary to said isolated polynucleotide
- and polynucleotides that are variants and fragments of the above mentioned polynucleotides or that are complementary to above mentioned polynucleotides, over the entire length thereof.
- 10
5. An isolated polynucleotide as claimed in claim 4 selected from the group consisting of:
- (a) an isolated polynucleotide comprising the polynucleotide of SEQ ID NO:1;
- (b) the isolated polynucleotide of SEQ ID NO:1;
- 15 (c) an isolated polynucleotide comprising a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2; and
- (d) an isolated polynucleotide encoding the polypeptide of SEQ ID NO:2.
6. An expression system comprising a polynucleotide capable of producing a
- 20 polypeptide of claim 1 when said expression vector is present in a compatible host cell.
7. A recombinant host cell comprising the expression vector of claim 6 or a membrane thereof expressing the polypeptide of claim 1.
- 25
8. A process for producing a polypeptide of claim 1 comprising the step of culturing a host cell as defined in claim 7 under conditions sufficient for the



production of said polypeptide and recovering the polypeptide from the culture medium.

9. A fusion protein consisting of the Immunoglobulin Fc-region and any one  
5 polypeptide of claim 1.

10. An antibody immunospecific for the polypeptide of any one of claims 1 to 3.

11. A method for screening to identify compounds that stimulate or inhibit the function or level of the polypeptide of claim 1 comprising a method selected from the group consisting of:

- 10 (a) measuring or, detecting, quantitatively or qualitatively, the binding of a candidate compound to the polypeptide (or to the cells or membranes expressing the polypeptide) or a fusion protein thereof by means of a label directly or indirectly associated with the candidate compound;
- (b) measuring the competition of binding of a candidate compound to the  
15 polypeptide (or to the cells or membranes expressing the polypeptide) or a fusion protein thereof in the presence of a labeled competitor;
- (c) testing whether the candidate compound results in a signal generated by activation or inhibition of the polypeptide, using detection systems appropriate to the cells or cell membranes expressing the polypeptide;
- 20 (d) mixing a candidate compound with a solution containing a polypeptide of claim 1, to form a mixture, measuring activity of the polypeptide in the mixture, and comparing the activity of the mixture to a control mixture which contains no candidate compound; or
- (e) detecting the effect of a candidate compound on the production of mRNA  
25 encoding said polypeptide or said polypeptide in cells, using for instance, an ELISA assay, and
- (f) producing said compound according to biotechnological or chemical standard techniques.



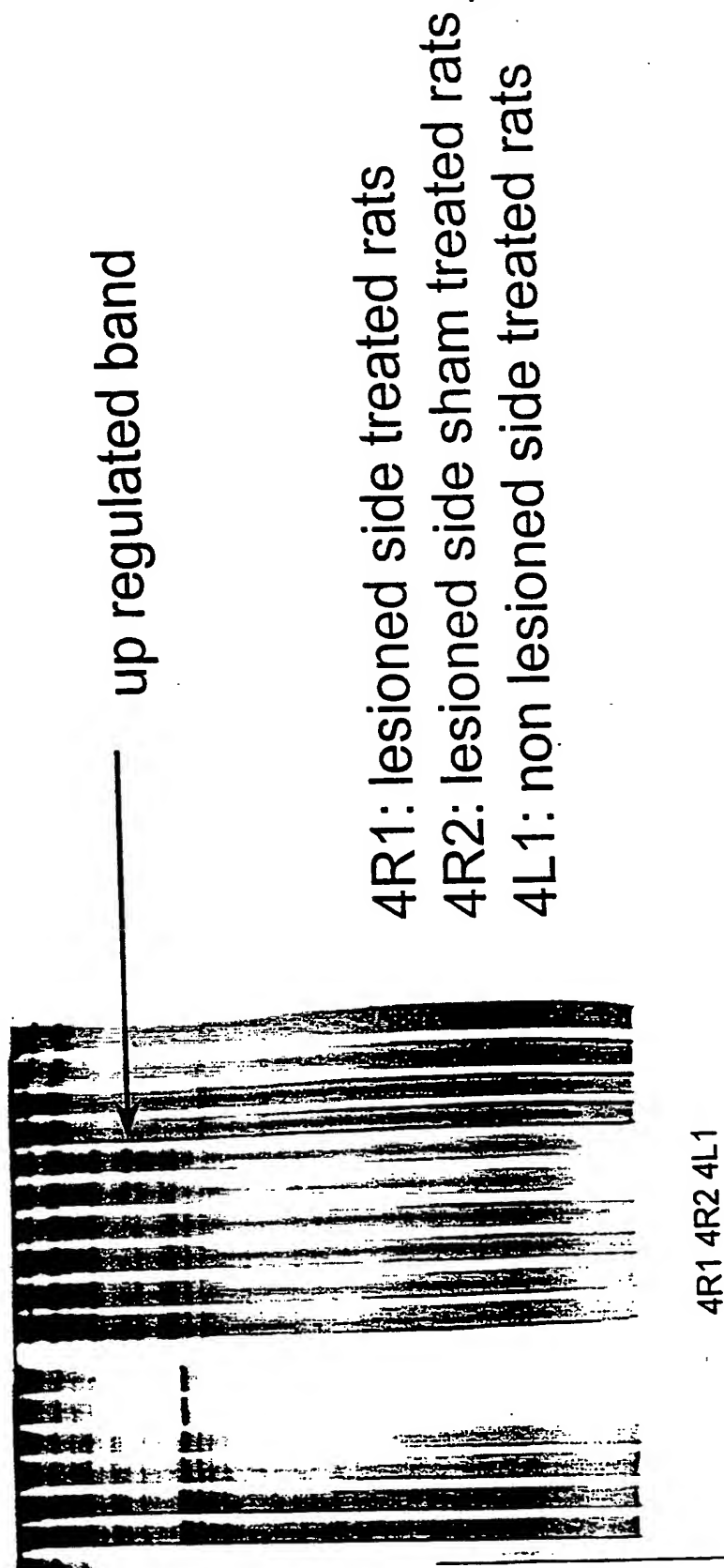


Fig.1





500 bp marker

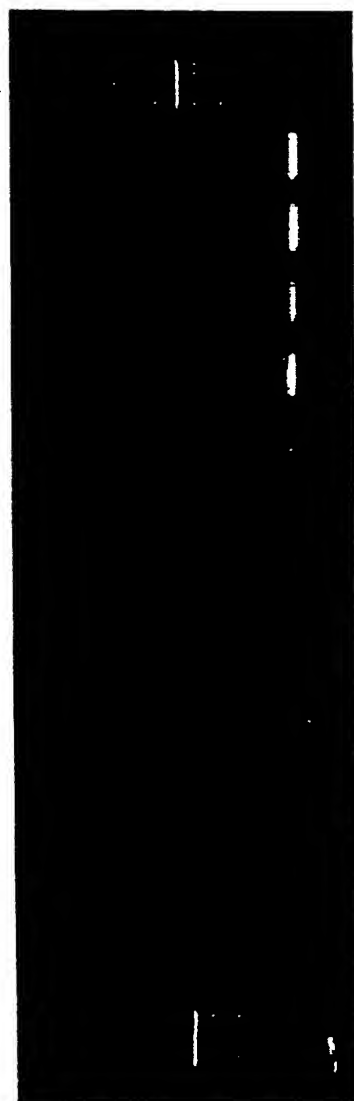


Fig.2



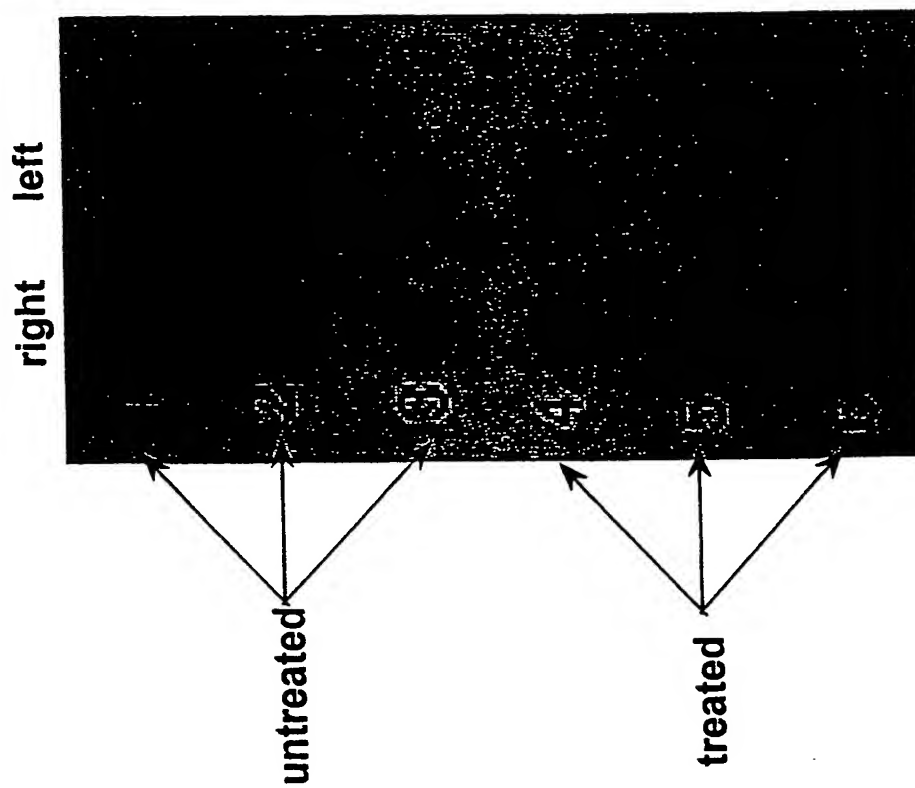


Fig.3



4/6

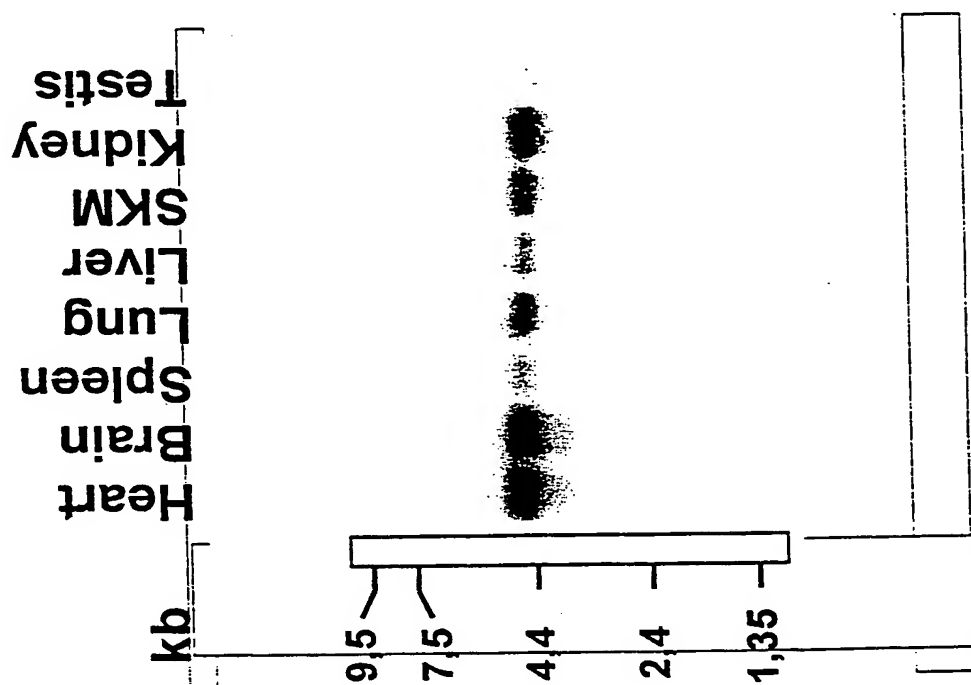
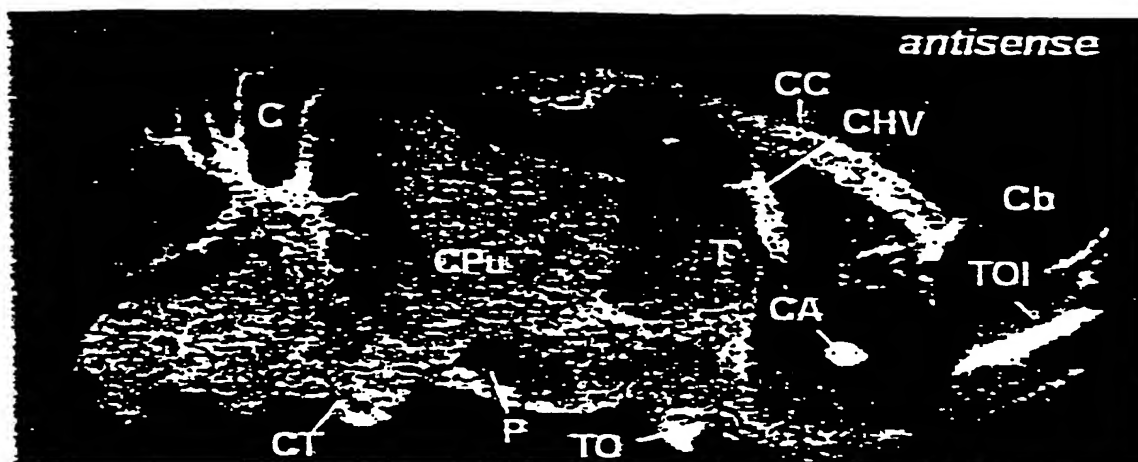


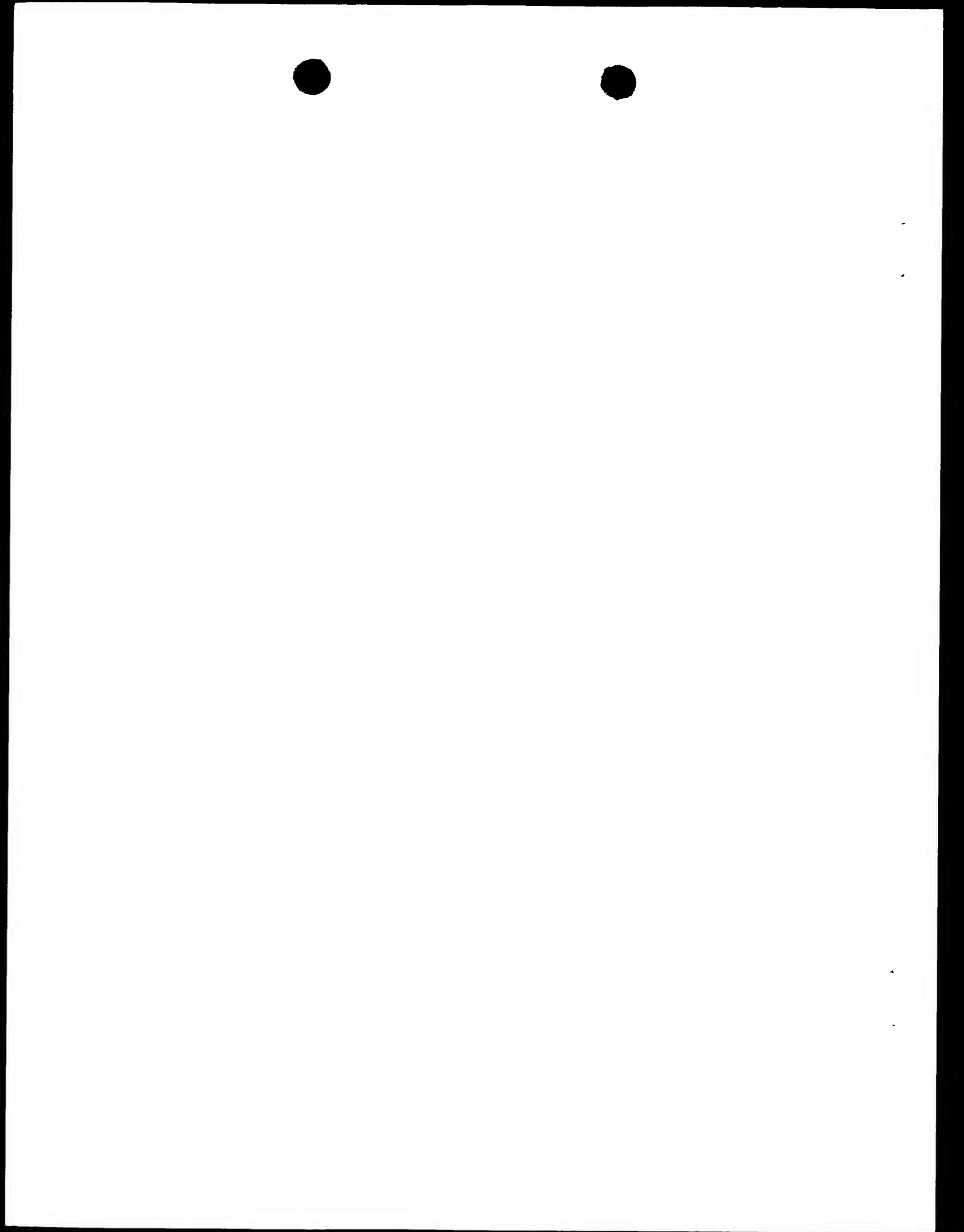
Fig.4



Fig. 5



- |     |                                 |     |                                 |
|-----|---------------------------------|-----|---------------------------------|
| C   | Cerebellum                      | CT  | Corpus trapezoideum             |
| CA  | Commissura anterior             | FL  | Fasciculus longitudinalis       |
| Cb  | Cerebrum                        | NT  | Nervus trigeminus               |
| CC  | Corpus callosum                 | P   | Pons                            |
| CHV | Commissura hippocampi ventralis | SM  | Stria medullaris thalami        |
| CI  | Colliculus inferior             | T   | Thalamus                        |
| CPu | Nucleus caudatus/putamen        | TO  | Tractus opticus                 |
| CS  | Colliculus superior             | TOI | Tractus olfactorius intermedius |





# Expression of ANIC-BP after Head Trauma

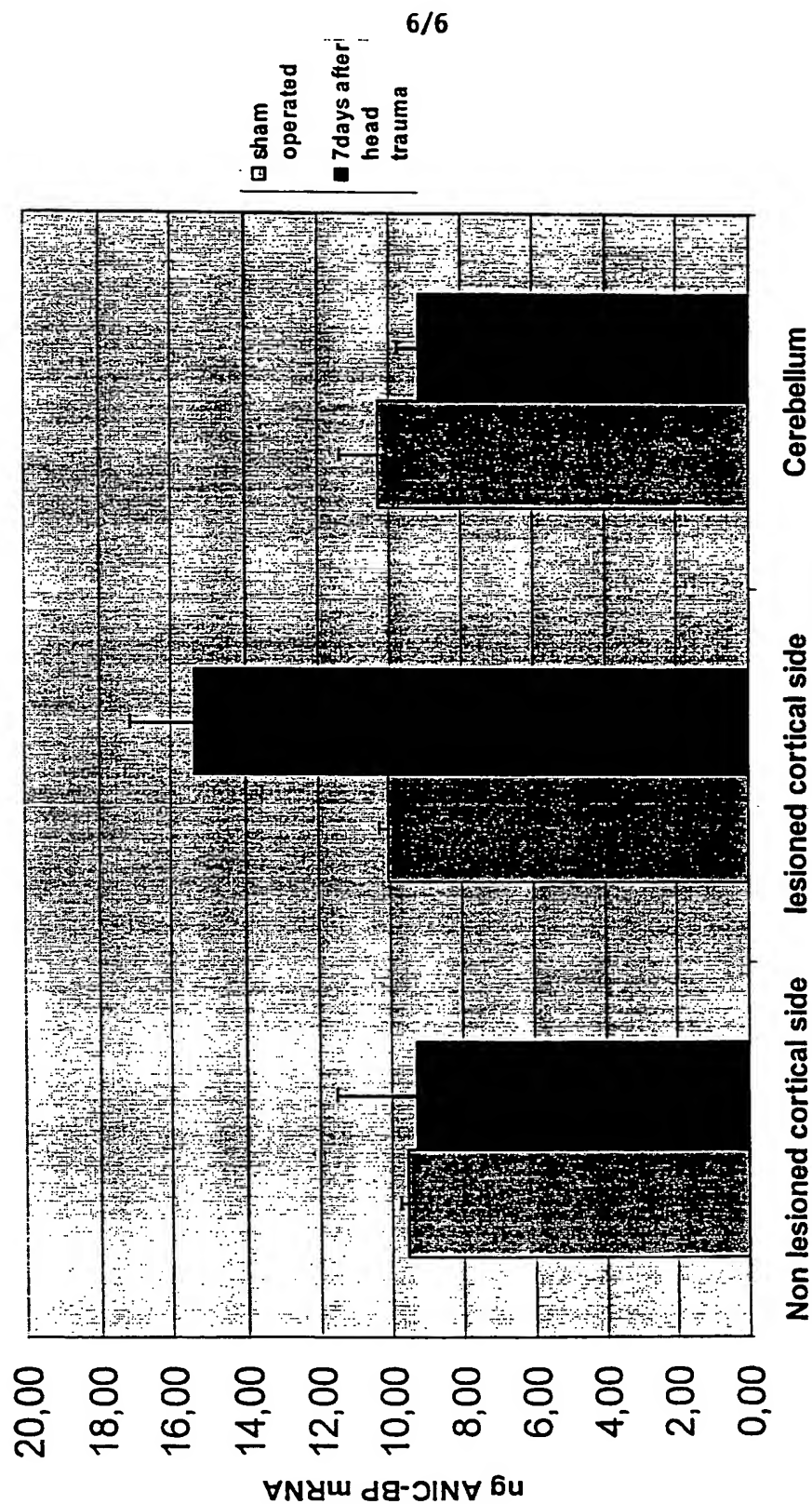


Fig. 6





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: **CALCIUM-BINDING PHOSPHOPROTEIN**

```

1  M A G T A R H D R E M A I O A K K K L T T A T D P I E R L R L Q C L A R G S A G CBPP-1
1  M D - - - - - A V D A T V E K L R A Q C L S R G A L G g877
1  M D - - - - - A V D A T M E K L R A Q C L S R G A S G g1359717

41  I K G L G R V F R I M D D D N N R T L D F K E F M K G L N D Y A V V M E K E E V CBPP-1
23  I Q G L A R F F R R L D R D S R S L D S R E L Q R G L A E L G L V L D T A E A g877
23  I Q G L A R F F R Q L D R D G S R S L D A D E F R Q G L A K L G L V L D Q A E A g1359717

81  E E L F R R F D K D G N G T I D F N E F L L T L R P P M S R A R K E V I M O A F CBPP-1
63  E G V C R R W D R D G S G T L D L E E F L R A L R P P M S Q A R E A V I A A A F g877
63  E G V C R K W D R N G S G T L D L E E F L R A L R P P M S Q A R E A V I A A A F g1359717

121 R K L D K T G D G V I T I E D L R E V Y N A K H H P K Y O N G E W S E E Q V E R CBPP-1
103 A K L D R S G D G V V T V D D L R G V Y S G R T H P K V Q S G E W T E E E V L R g877
103 A K L D R S G D G V V T V D D L R G V Y S G R A H P K V R S G E W T E D E V L R g1359717

161 K F L D N F D S P Y D K D G L V T P E E F M N Y Y A G V S A S I D T D V Y F I I CBPP-1
143 R F L D N F D S S - E K D G Q V T L A E F Q D Y Y S G V S A S M D T D E E F V A g877
143 R F L D N F D S S - E K D G Q V T L A E F Q D Y Y S G V S A S M N T D E E F V A g1359717

201 M M R T A W K L CBPP-1
182 M M T S A W Q I g877
182 M M T S A W Q I g1359717

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## (57) Abstract

The invention provides a human calcium-binding phosphoprotein (CBPP-1) and polynucleotides which identify and encode CBPP-1. The invention also provides expression vectors, host cells, agonists, antibodies and antagonists. The invention also provides methods for treating disorders associated with expression of CBPP-1.

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## CALCIUM-BINDING PHOSPHOPROTEIN

### TECHNICAL FIELD

This invention relates to nucleic acid and amino acid sequences of a calcium-binding phosphoprotein and to the use of these sequences in the diagnosis, prevention, and treatment of neurological and developmental disorders.

5

### BACKGROUND OF THE INVENTION

Calcium functions as an intracellular mediator of a variety of physiological activities in cells. These activities include gene regulation, DNA synthesis, cell cycle regulation, signal transduction, release of neurotransmitters in the brain, and the breakdown of glycogen for muscle contraction. These effects are initiated when extracellular signals trigger the release of calcium either from the extracellular space or the endoplasmic reticulum into the cytosol. There, calcium binds to a variety of calcium-binding proteins that further mediate the signal by activating other molecules leading to a particular physiological effect.

Calcium-binding proteins (CBP) are a super family of proteins related by the presence of a calcium-binding motif referred to as the "EF-hand" domain. This domain is characterized by a 12 amino acid loop flanked by two alpha-helices oriented at approximately 90° to one another (Celio, M.R. et al. (1996) Guidebook to Calcium-binding Proteins, Oxford University Press, Oxford, UK, pp. 15-20). Most CBPs have multiple EF-hand motifs for binding calcium, and more than 250 such CBPs have been described.

Calmodulin (CaM) is the most widely distributed and the most common mediator of calcium effects. CaM contains four EF-hand domains and undergoes a conformational change when it binds calcium. Activation of CaM enables it to bind to other target proteins and alter their activity. Key targets of CaM are the CaM-dependent protein kinases that are involved in regulation of smooth muscle contraction, glycogen breakdown, and neurotransmission, and calcineuron that is involved in synaptic transmission in the brain.

Calcyphosine is another CBP that is regulated by both calcium binding and protein phosphorylation. Dog calcyphosine (p24) is a CBP which has three EF-hand domains and is phosphorylated by cyclic-AMP dependent protein kinase (Lefort, A. et al. (1989) *EMBO* 8:111-116). The exact function of p24 is unknown; however, its occurrence in various secretory tissues such as salivary glands, lung, and brain suggests that it may play a role in the regulation of ionic transport (Celio et al, supra). A similar calcium-binding phosphoprotein from rabbit,

R2D5, is expressed predominantly in and may modulate signal transduction in olfactory neurons. R2D5 also has three EF-hand domains and is phosphorylated by both cAMP-dependent protein kinase and CaM-kinase (Nemoto Y. et al. (1993) J. Cell Biol. 123:963-76).

The regulation of CBPs has implications for the control of a variety of disease conditions.

5 The immunosuppressive agents cyclosporin and FK506 appear to act in part by inhibiting calcineuron mediated T-cell activation. Such inhibition indicates the importance of calcineuron, and hence CaM, in the immune response (Schwaninger M. et al. (1993) J. Biol Chem. 268:23111-15). Calcineuron also appears to be important for synaptic transmission in the brain and may be involved in learning and memory disorders (Mulkey R.M. et al. (1993) Science  
10 261:1051-55). Since CaM-kinases are involved in muscle contraction and neurotransmission, they may play a role in muscular and neurological disorders.

The discovery of a new calcium-binding phosphoprotein and the polynucleotides encoding it satisfies a need in the art by providing new compositions which are useful in the diagnosis, prevention and treatment of neurological and developmental disorders.

15

### SUMMARY OF THE INVENTION

The invention features a substantially purified polypeptide, calcium-binding phosphoprotein (CBPP-1), having the amino acid sequence shown in SEQ ID NO:1, or fragments thereof.

20 The invention further provides an isolated and substantially purified polynucleotide sequence encoding the polypeptide comprising the amino acid sequence of SEQ ID NO:1 or fragments thereof and a composition comprising said polynucleotide sequence. The invention also provides a polynucleotide sequence which hybridizes under stringent conditions to the polynucleotide sequence encoding the amino acid sequence SEQ ID NO:1, or fragments of said  
25 polynucleotide sequence. The invention further provides a polynucleotide sequence comprising the complement of the polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:1, or fragments or variants of said polynucleotide sequence.

The invention also provides an isolated and purified sequence comprising SEQ ID NO.2 or variants thereof. In addition, the invention provides a polynucleotide sequence which  
30 hybridizes under stringent conditions to the polynucleotide sequence of SEQ ID NO:2. In another aspect the invention provides a composition comprising an isolated and purified polynucleotide sequence comprising the complement of SEQ ID NO:2, or fragments or variants

thereof. The invention also provides a polynucleotide sequence comprising the complement of SEQ ID NO:2.

The present invention further provides an expression vector containing at least a fragment of any of the claimed polynucleotide sequences. In yet another aspect, the expression vector  
5 containing the polynucleotide sequence is contained within a host cell.

The invention also provides a method for producing a polypeptide comprising the amino acid sequence of SEQ ID NO:1 or a fragment thereof, the method comprising the steps of: a) culturing the host cell containing an expression vector containing at least a fragment of the polynucleotide sequence encoding CBPP-1 under conditions suitable for the expression of the  
10 polypeptide; and b) recovering the polypeptide from the host cell culture.

The invention also provides a pharmaceutical composition comprising a substantially purified CBPP-1 having the amino acid sequence of SEQ ID NO:1 in conjunction with a suitable pharmaceutical carrier.

The invention also provides a purified antagonist of a polypeptide of SEQ ID NO:1. In  
15 one aspect the invention provides a purified antibody which binds to a polypeptide comprising at least a fragment of the amino acid sequence of SEQ ID NO:1.

Still further, the invention provides a purified agonist of the polypeptide of SEQ ID NO:1.

The invention also provides a method for treating or preventing a neurological disorder comprising administering to a subject in need of such treatment an effective amount of a  
20 pharmaceutical composition comprising purified CBPP-1.

The invention also provides a method for treating or preventing a developmental disorder comprising administering to a subject in need of such treatment an effective amount of a pharmaceutical composition comprising purified CBPP-1.

The invention also provides a method for detecting a polynucleotide which encodes  
25 CBPP-1 in a biological sample comprising the steps of: a) hybridizing a polynucleotide sequence complementary to the polynucleotide encoding CBPP-1 (SEQ ID NO:1) to nucleic acid material of a biological sample, thereby forming a hybridization complex; and b) detecting the hybridization complex, wherein the presence of the complex correlates with the presence of a polynucleotide encoding CBPP-1 in the biological sample. In a preferred embodiment, prior to  
30 hybridization, the nucleic acid material of the biological sample is amplified by the polymerase chain reaction prior to hybridization.

## BRIEF DESCRIPTION OF THE FIGURES

Figures 1A, 1B, and 1C show the amino acid sequence (SEQ ID NO:1) and nucleic acid sequence (SEQ ID NO:2) of CBPP-1. The alignment was produced using MAC DNASIS PRO™ software (Hitachi Software Engineering Co. Ltd. San Bruno, CA).

5 Figure 2 shows the amino acid sequence alignments among CBPP-1 (SEQ ID NO:1), and the calcium-binding phosphoprotein, calcyphosine, from dog, (GI 877; SEQ ID NO:3) and human, (GI 1359717; SEQ ID NO:4), produced using the multisequence alignment program of DNASTAR™ software (DNASTAR Inc, Madison WI).

10 Figures 3A, 3B, and 3C show the hydrophobicity plots for CBPP-1, SEQ ID NO: 1 and calcyphosine from dog (SEQ ID NO:3), and human (SEQ ID NO:4), respectively ; the positive X axis reflects amino acid position, and the negative Y axis, hydrophobicity (MAC DNASIS PRO software).

## DESCRIPTION OF THE INVENTION

15 Before the present proteins, nucleotide sequences, and methods are described, it is understood that this invention is not limited to the particular methodology, protocols, cell lines, vectors, and reagents described, as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended  
20 claims.

It must be noted that as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to “a host cell” includes a plurality of such host cells, reference to the “antibody” is a reference to one or more antibodies and equivalents thereof known to those  
25 skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and  
30 materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies which are reported in the publications which might be used in connection with the invention.



Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

## DEFINITIONS

5 CBPP-1, as used herein, refers to the amino acid sequences of substantially purified CBPP-1 obtained from any species, particularly mammalian, including bovine, ovine, porcine, murine, equine, and preferably human, from any source whether natural, synthetic, semi-synthetic, or recombinant.

The term "agonist", as used herein, refers to a molecule which, when bound to CBPP-1, 10 increases or prolongs the duration of the effect of CBPP-1. Agonists may include proteins, nucleic acids, carbohydrates, or any other molecules which bind to and modulate the effect of CBPP-1.

An "allele" or "allelic sequence", as used herein, is an alternative form of the gene encoding CBPP-1. Alleles may result from at least one mutation in the nucleic acid sequence 15 and may result in altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given natural or recombinant gene may have none, one, or many allelic forms. Common mutational changes which give rise to alleles are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

20 "Altered" nucleic acid sequences encoding CBPP-1 as used herein include those with deletions, insertions, or substitutions of different nucleotides resulting in a polynucleotide that encodes the same or a functionally equivalent CBPP-1. Included within this definition are polymorphisms which may or may not be readily detectable using a particular oligonucleotide probe of the polynucleotide encoding CBPP-1, and improper or unexpected hybridization to 25 alleles, with a locus other than the normal chromosomal locus for the polynucleotide sequence encoding CBPP-1. The encoded protein may also be "altered" and contain deletions, insertions, or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent CBPP-1. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of 30 the residues as long as the biological or immunological activity of CBPP-1 is retained. For example, negatively charged amino acids may include aspartic acid and glutamic acid; positively charged amino acids may include lysine and arginine; and amino acids with uncharged polar head

groups having similar hydrophilicity values may include leucine, isoleucine, and valine, glycine and alanine, asparagine and glutamine, serine and threonine, and phenylalanine and tyrosine.

"Amino acid sequence" as used herein refers to an oligopeptide, peptide, polypeptide, or protein sequence, and fragment thereof, and to naturally occurring or synthetic molecules.

5 Fragments of CBPP-1 are preferably about 5 to about 15 amino acids in length and retain the biological activity or the immunological activity of CBPP-1. Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, amino acid sequence, and like terms, are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

10 "Amplification" as used herein refers to the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) technologies well known in the art (Dieffenbach, C.W. and G.S. Dveksler (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, NY).

The term "antagonist" as used herein, refers to a molecule which, when bound to CBPP-1, 15 decreases the amount or the duration of the effect of the biological or immunological activity of CBPP-1. Antagonists may include proteins, nucleic acids, carbohydrates, or any other molecules which decrease the effect of CBPP-1.

As used herein, the term "antibody" refers to intact molecules as well as fragments thereof, such as Fa, F(ab')<sub>2</sub>, and Fv, which are capable of binding the epitopic determinant.

20 Antibodies that bind CBPP-1 polypeptides can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or oligopeptide used to immunize an animal can be derived from the translation of RNA or synthesized chemically and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin, keyhole limpet 25 hemocyanin. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

The term "antigenic determinant", as used herein, refers to that fragment of a molecule (i.e., an epitope) that makes contact with a particular antibody. When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the 30 production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

The term "antisense", as used herein, refers to any composition containing nucleotide sequences which are complementary to a specific DNA or RNA sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. Antisense molecules include peptide nucleic acids and may be produced by any method including synthesis or transcription. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form duplexes and block either transcription or translation. The designation "negative" is sometimes used in reference to the antisense strand, and "positive" is sometimes used in reference to the sense strand.

The term "biologically active", as used herein, refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic CBPP-1, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

The terms "complementary" or "complementarity", as used herein, refer to the natural binding of polynucleotides under permissive salt and temperature conditions by base-pairing. For example, the sequence "A-G-T" binds to the complementary sequence "T-C-A". Complementarity between two single-stranded molecules may be "partial", in which only some of the nucleic acids bind, or it may be complete when total complementarity exists between the single stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, which depend upon binding between nucleic acids strands and in the design and use of PNA molecules.

A "composition comprising a given polynucleotide sequence" as used herein refers broadly to any composition containing the given polynucleotide sequence. The composition may comprise a dry formulation or an aqueous solution. Compositions comprising polynucleotide sequences encoding CBPP-1 (SEQ ID NO:1) or fragments thereof (e.g., SEQ ID NO:2 and fragments thereof) may be employed as hybridization probes. The probes may be stored in freeze-dried form and may be associated with a stabilizing agent such as a carbohydrate. In hybridizations, the probe may be deployed in an aqueous solution containing salts (e.g., NaCl), detergents (e.g., SDS) and other components (e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.).

"Consensus", as used herein, refers to a nucleic acid sequence which has been resequenced to resolve uncalled bases, has been extended using XL-PCR™ (Perkin Elmer,

Norwalk, CT) in the 5' and/or the 3' direction and resequenced, or has been assembled from the overlapping sequences of more than one Incyte Clone using a computer program for fragment assembly (e.g., GELVIEW™ Fragment Assembly system, GCG, Madison, WI). Some sequences have been both extended and assembled to produce the consensus sequence .

5       The term "correlates with expression of a polynucleotide", as used herein, indicates that the detection of the presence of ribonucleic acid that is similar to SEQ ID NO:2 by northern analysis is indicative of the presence of mRNA encoding CBPP-1 in a sample and thereby correlates with expression of the transcript from the polynucleotide encoding the protein.

10       A "deletion", as used herein, refers to a change in the amino acid or nucleotide sequence and results in the absence of one or more amino acid residues or nucleotides.

15       The term "derivative", as used herein, refers to the chemical modification of a nucleic acid encoding or complementary to CBPP-1 or the encoded CBPP-1. Such modifications include, for example, replacement of hydrogen by an alkyl, acyl, or amino group. A nucleic acid derivative encodes a polypeptide which retains the biological or immunological function of the natural molecule. A derivative polypeptide is one which is modified by glycosylation, pegylation, or any similar process which retains the biological or immunological function of the polypeptide from which it was derived.

20       The term "homology", as used herein, refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or hybridization probe will compete for and inhibit the binding of a completely homologous sequence to the target sequence under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% identity). In the absence of non-specific binding, the probe will not hybridize to the second non-complementary target sequence.

30

Human artificial chromosomes (HACs) are linear microchromosomes which may contain DNA sequences of 10K to 10M in size and contain all of the elements required for stable mitotic chromosome segregation and maintenance (Harrington, J.J. et al. (1997) Nat Genet. 15:345-355).

The term "humanized antibody", as used herein, refers to antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a human antibody, while still retaining the original binding ability.

The term "hybridization", as used herein, refers to any process by which a strand of nucleic acid binds with a complementary strand through base pairing.

The term "hybridization complex", as used herein, refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bonds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (e.g.,  $C_0t$  or  $R_0t$  analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized on a solid support (e.g., paper, membranes, filters, chips, pins or glass slides, or any other appropriate substrate to which cells or their nucleic acids have been fixed).

An "insertion" or "addition", as used herein, refers to a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, as compared to the naturally occurring molecule.

"Microarray" refers to an array of distinct polynucleotides or oligonucleotides synthesized on a substrate, such as paper, nylon or other type of membrane, filter, chip, glass slide, or any other suitable solid support.

The term "modulate", as used herein, refers to a change in the activity of CBPP-1. For example, modulation may cause an increase or a decrease in protein activity, binding characteristics, or any other biological, functional or immunological properties of CBPP-1.

"Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide, or polynucleotide, and fragments thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. "Fragments" are those nucleic acid sequences which are greater than 60 nucleotides in length, and most preferably includes fragments that are at least 100 nucleotides or at least 1000 nucleotides, and at least 10,000 nucleotides in length.

The term "oligonucleotide" refers to a nucleic acid sequence of at least about 6 nucleotides to about 60 nucleotides, preferably about 15 to 30 nucleotides, and more preferably about 20 to 25 nucleotides, which can be used in PCR amplification or hybridization assays. As used herein, oligonucleotide is substantially equivalent to the terms "amplimers", "primers",  
5 "oligomers", and "probes", as commonly defined in the art.

"Peptide nucleic acid", PNA as used herein, refers to an antisense molecule or anti-gene agent which comprises an oligonucleotide of at least five nucleotides in length linked to a peptide backbone of amino acid residues which ends in lysine. The terminal lysine confers solubility to the composition. PNAs may be pegylated to extend their lifespan in the cell where they  
10 preferentially bind complementary single stranded DNA and RNA and stop transcript elongation (Nielsen, P.E. et al. (1993) Anticancer Drug Des. 8:53-63).

The term "portion", as used herein, with regard to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from five amino acid residues to the entire amino acid sequence minus one amino acid. Thus, a protein  
15 "comprising at least a portion of the amino acid sequence of SEQ ID NO:1" encompasses the full-length CBPP-1 and fragments thereof.

The term "sample", as used herein, is used in its broadest sense. A biological sample suspected of containing nucleic acid encoding CBPP-1, or fragments thereof, or CBPP-1 itself may comprise a bodily fluid, extract from a cell, chromosome, organelle, or membrane isolated  
20 from a cell, a cell, genomic DNA, RNA, or cDNA(in solution or bound to a solid support, a tissue, a tissue print, and the like.

The terms "specific binding" or "specifically binding", as used herein, refers to that interaction between a protein or peptide and an agonist, an antibody and an antagonist. The interaction is dependent upon the presence of a particular structure (i.e., the antigenic determinant  
25 or epitope) of the protein recognized by the binding molecule. For example, if an antibody is specific for epitope "A", the presence of a protein containing epitope A (or free, unlabeled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

The terms "stringent conditions" or "stringency", as used herein, refer to the conditions for  
30 hybridization as defined by the nucleic acid, salt, and temperature. These conditions are well known in the art and may be altered in order to identify or detect identical or related polynucleotide sequences. Numerous equivalent conditions comprising either low or high stringency depend on factors such as the length and nature of the sequence (DNA, RNA, base

composition), nature of the target (DNA, RNA, base composition), milieu (in solution or immobilized on a solid substrate), concentration of salts and other components (e.g., formamide, dextran sulfate and/or polyethylene glycol), and temperature of the reactions (within a range from about 5°C below the melting temperature of the probe to about 20°C to 25°C below the melting temperature). One or more factors be may be varied to generate conditions of either low or high stringency different from, but equivalent to, the above listed conditions.

The term "substantially purified", as used herein, refers to nucleic or amino acid sequences that are removed from their natural environment, isolated or separated, and are at least 60% free, preferably 75% free, and most preferably 90% free from other components with which they are naturally associated.

A "substitution", as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

"Transformation", as defined herein, describes a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the type of host cell being transformed and may include, but is not limited to, viral infection, electroporation, heat shock, lipofection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. They also include cells which transiently express the inserted DNA or RNA for limited periods of time.

A "variant" of CBPP-1, as used herein, refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art, for example, DNASTAR software.

**THE INVENTION**

The invention is based on the discovery of a new human calcium-binding phosphoprotein (hereinafter referred to as "CBPP-1"), the polynucleotides encoding CBPP-1, and the use of these compositions for the diagnosis, prevention, or treatment of neurological and developmental disorders.

Nucleic acids encoding the CBPP-1 of the present invention were first identified in Incyte Clone 1850226 (SEQ ID NO:2) from the fetal lung tissue cDNA library (LUNGFET03) using a computer search for amino acid sequence alignments.

In one embodiment, the invention encompasses a polypeptide comprising the amino acid sequence of SEQ ID NO:1, as shown in Figure 1. CBPP-1 is 208 amino acids in length and has two potential EF-hand calcium-binding domains at D<sub>52</sub>DDNNRTLDFKEF and D<sub>88</sub>KDGNGTIDFNEF. CBPP-1 also contains various potential protein kinase phosphorylation sites, including a cAMP-dependent protein kinase site at T<sub>20</sub>, three casein kinase II phosphorylation sites at T<sub>132</sub>, S<sub>168</sub>, and T<sub>177</sub>, and two protein kinase C phosphorylation sites at T<sub>4</sub> and T<sub>103</sub>. As shown in Figure 2, CBPP-1 has chemical and structural homology with calcyphosine from dog (GI 877; SEQ ID NO:3) and human (GI 1359717). In particular, CBPP-1 shares 55% and 53% identity with dog and human calcyphosine, respectively. The dog and human calcyphosine both share the two EF-hand domains found in CBPP-1. CBPP-1 is distinguished by the presence of an N-terminal sequence extending from M<sub>1</sub> to I<sub>26</sub> that may represent a signal peptide directing CBPP-1 to a different sub-cellular location. As illustrated by Figure 3, CBPP-1 and the dog and human calcyphosines have rather similar hydrophobicity plots. All three proteins are primarily hydrophilic with a region of hydrophobicity at the C-terminus. Northern analysis shows the expression of this sequence in libraries associated with the brain (epilepsy) and fetal lung.

The invention also encompasses CBPP-1 variants. A preferred CBPP-1 variant is one having at least 80%, and more preferably 90%, amino acid sequence identity to the CBPP-1 amino acid sequence (SEQ ID NO:1) and which retains at least one biological, structural, or other functional characteristic of CBPP-1. A most preferred CBPP-1 variant is one having at least 95% amino acid sequence identity to SEQ ID NO:1.

The invention also encompasses polynucleotides which encode CBPP-1. Accordingly, any nucleic acid sequence which encodes the amino acid sequence of CBPP-1 can be used to produce recombinant molecules which express CBPP-1. In a particular embodiment, the



invention encompasses the polynucleotide comprising the nucleic acid sequence of SEQ ID NO:2 as shown in Figure 1.

It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding CBPP-1, some bearing minimal  
5 homology to the nucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention contemplates each and every possible variation of nucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence of naturally occurring CBPP-1, and all such variations are to be considered  
10 as being specifically disclosed.

Although nucleotide sequences which encode CBPP-1 and its variants are preferably capable of hybridizing to the nucleotide sequence of the naturally occurring CBPP-1 under appropriately selected conditions of stringency, it may be advantageous to produce nucleotide sequences encoding CBPP-1 or its derivatives possessing a substantially different codon usage.

15 Codons may be selected to increase the rate at which expression of the peptide occurs in a particular prokaryotic or eukaryotic host in accordance with the frequency with which particular codons are utilized by the host. Other reasons for substantially altering the nucleotide sequence encoding CBPP-1 and its derivatives without altering the encoded amino acid sequences include the production of RNA transcripts having more desirable properties, such as a greater half-life,  
20 than transcripts produced from the naturally occurring sequence.

The invention also encompasses production of DNA sequences, or fragments thereof, which encode CBPP-1 and its derivatives, entirely by synthetic chemistry. After production, the synthetic sequence may be inserted into any of the many available expression vectors and cell systems using reagents that are well known in the art. Moreover, synthetic chemistry may be  
25 used to introduce mutations into a sequence encoding CBPP-1 or any fragment thereof.

Also encompassed by the invention are polynucleotide sequences that are capable of hybridizing to the claimed nucleotide sequences, and in particular, those shown in SEQ ID NO:2, under various conditions of stringency as taught in Wahl, G.M. and S.L. Berger (1987; Methods Enzymol. 152:399-407) and Kimmel, A.R. (1987; Methods Enzymol. 152:507-511).

30 Methods for DNA sequencing which are well known and generally available in the art and may be used to practice any of the embodiments of the invention. The methods may employ such enzymes as the Klenow fragment of DNA polymerase I, SEQUENASE® (US Biochemical Corp, Cleveland, OH), Taq polymerase (Perkin Elmer), thermostable T7 polymerase (Amersham,

Chicago, IL), or combinations of polymerases and proofreading exonucleases such as those found in the ELONGASE Amplification System marketed by Gibco/BRL (Gaithersburg, MD).

Preferably, the process is automated with machines such as the Hamilton Micro Lab 2200 (Hamilton, Reno, NV), Peltier Thermal Cycler (PTC200; MJ Research, Watertown, MA) and the  
5 ABI Catalyst and 373 and 377 DNA Sequencers (Perkin Elmer).

The nucleic acid sequences encoding CBPP-1 may be extended utilizing a partial nucleotide sequence and employing various methods known in the art to detect upstream sequences such as promoters and regulatory elements. For example, one method which may be employed, "restriction-site" PCR, uses universal primers to retrieve unknown sequence adjacent  
10 to a known locus (Sarkar, G. (1993) PCR Methods Applic. 2:318-322). In particular, genomic DNA is first amplified in the presence of primer to a linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse  
15 transcriptase.

Inverse PCR may also be used to amplify or extend sequences using divergent primers based on a known region (Triglia, T. et al. (1988) Nucleic Acids Res. 16:8186). The primers may be designed using commercially available software such as OLIGO 4.06 Primer Analysis software (National Biosciences Inc., Plymouth, MN), or another appropriate program, to be 22-30  
20 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68°-72° C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Another method which may be used is capture PCR which involves PCR amplification of  
25 DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA (Lagerstrom, M. et al. (1991) PCR Methods Applic. 1:111-119). In this method, multiple restriction enzyme digestions and ligations may also be used to place an engineered double-stranded sequence into an unknown fragment of the DNA molecule before performing PCR.

Another method which may be used to retrieve unknown sequences is that of Parker, J.D. et al. (1991; Nucleic Acids Res. 19:3055-3060). Additionally, one may use PCR, nested primers, and PROMOTER FINDER™ libraries to walk genomic DNA (Clontech, Palo Alto, CA). This  
30 process avoids the need to screen libraries and is useful in finding intron/exon junctions.

When screening for full-length cDNAs, it is preferable to use libraries that have been size-selected to include larger cDNAs. Also, random-primed libraries are preferable, in that they will contain more sequences which contain the 5' regions of genes. Use of a randomly primed library may be especially preferable for situations in which an oligo d(T) library does not yield a full-length cDNA. Genomic libraries may be useful for extension of sequence into 5' non-transcribed regulatory regions.

Capillary electrophoresis systems which are commercially available may be used to analyze the size or confirm the nucleotide sequence of sequencing or PCR products. In particular, capillary sequencing may employ flowable polymers for electrophoretic separation, four different fluorescent dyes (one for each nucleotide) which are laser activated, and detection of the emitted wavelengths by a charge coupled device camera. Output/light intensity may be converted to electrical signal using appropriate software (e.g. GENOTYPER™ and SEQUENCE NAVIGATOR™, Perkin Elmer) and the entire process from loading of samples to computer analysis and electronic data display may be computer controlled. Capillary electrophoresis is especially preferable for the sequencing of small pieces of DNA which might be present in limited amounts in a particular sample.

In another embodiment of the invention, polynucleotide sequences or fragments thereof which encode CBPP-1 may be used in recombinant DNA molecules to direct expression of CBPP-1, fragments or functional equivalents thereof, in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be produced, and these sequences may be used to clone and express CBPP-1.

As will be understood by those of skill in the art, it may be advantageous to produce CBPP-1-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce an RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

The nucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter CBPP-1 encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the gene product. DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. For

example, site-directed mutagenesis may be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, introduce mutations, and so forth.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences encoding CBPP-1 may be ligated to a heterologous sequence to encode a fusion protein. For example, to screen peptide libraries for inhibitors of CBPP-1 activity, it may be useful to encode a chimeric CBPP-1 protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site located between the CBPP-1 encoding sequence and the heterologous protein sequence, so that CBPP-1 may be cleaved and purified away from the heterologous moiety.

In another embodiment, sequences encoding CBPP-1 may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers, M.H. et al. (1980) Nucl. Acids Res. Symp. Ser. 215-223, Horn, T. et al. (1980) Nucl. Acids Res. Symp. Ser. 225-232). Alternatively, the protein itself may be produced using chemical methods to synthesize the amino acid sequence of CBPP-1, or a fragment thereof. For example, peptide synthesis can be performed using various solid-phase techniques (Roberge, J.Y. et al. (1995) Science 269:202-204) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer).

The newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography (e.g., Creighton, T. (1983) Proteins, Structures and Molecular Principles, WH Freeman and Co., New York, NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, supra). Additionally, the amino acid sequence of CBPP-1, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

In order to express a biologically active CBPP-1, the nucleotide sequences encoding CBPP-1 or functional equivalents, may be inserted into appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence.

Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding CBPP-1 and appropriate transcriptional and translational control elements. These methods include in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. Such techniques are described in Sambrook, J. et al. (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press,

Plainview, NY, and Ausubel, F.M. et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY.

A variety of expression vector/host systems may be utilized to contain and express sequences encoding CBPP-1. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression  
5 vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems.

10 The invention is not limited by the host cell employed.

The "control elements" or "regulatory sequences" are those non-translated regions of the vector--enhancers, promoters, 5' and 3' untranslated regions--which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable  
15 transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT® phagemid (Stratagene, LaJolla, CA) or pSport1™ plasmid (Gibco BRL) and the like may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock,  
20 RUBISCO; and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding CBPP-1, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

25 In bacterial systems, a number of expression vectors may be selected depending upon the use intended for CBPP-1. For example, when large quantities of CBPP-1 are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be used. Such vectors include, but are not limited to, the multifunctional E. coli cloning and expression vectors such as BLUESCRIPT® (Stratagene), in which the sequence  
30 encoding CBPP-1 may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of  $\beta$ -galactosidase so that a hybrid protein is produced; pIN vectors (Van Heeke, G. and S.M. Schuster (1989) J. Biol. Chem. 264:5503-5509); and the like. pGEX vectors (Promega, Madison, WI) may also be used to express foreign polypeptides as

fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast, Saccharomyces cerevisiae, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. For reviews, see Ausubel et al. (supra) and Grant et al. (1987) *Methods Enzymol.* 153:516-544.

In cases where plant expression vectors are used, the expression of sequences encoding CBPP-1 may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) *EMBO J.* 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi, G. et al. (1984) *EMBO J.* 3:1671-1680; Broglie, R. et al. (1984) *Science* 224:838-843; and Winter, J. et al. (1991) *Results Probl. Cell Differ.* 17:85-105). These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (see, for example, Hobbs, S. or Murry, L.E. in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York, NY; pp. 191-196.

An insect system may also be used to express CBPP-1. For example, in one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in Spodoptera frugiperda cells or in Trichoplusia larvae. The sequences encoding CBPP-1 may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of CBPP-1 will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses may then be used to infect, for example, S. frugiperda cells or Trichoplusia larvae in which CBPP-1 may be expressed (Engelhard, E.K. et al. (1994) *Proc. Nat. Acad. Sci.* 91:3224-3227).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding CBPP-1 may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing CBPP-1 in infected host cells

(Logan, J. and Shenk, T. (1984) *Proc. Natl. Acad. Sci.* 81:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6 to 10M are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding CBPP-1. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding CBPP-1, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) *Results Probl. Cell Differ.* 20:125-162).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC; Bethesda, MD) and may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express CBPP-1 may be transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before

they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

5 Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. (1977) Cell 11:223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1980) Cell 22:817-23) genes which can be employed in tk<sup>-</sup> or apt<sup>-</sup> cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which  
10 confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. et al (1981) J. Mol. Biol. 150:1-14) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or  
15 hisD, which allows cells to utilize histinol in place of histidine (Hartman, S.C. and R.C. Mulligan (1988) Proc. Natl. Acad. Sci. 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins,  $\beta$  glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific  
20 vector system (Rhodes, C.A. et al. (1995) Methods Mol. Biol. 55:121-131).

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression may need to be confirmed. For example, if the sequence encoding CBPP-1 is inserted within a marker gene sequence, transformed cells containing sequences encoding CBPP-1 can be identified by the absence of marker gene function.  
25 Alternatively, a marker gene can be placed in tandem with a sequence encoding CBPP-1 under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

Alternatively, host cells which contain the nucleic acid sequence encoding CBPP-1 and express CBPP-1 may be identified by a variety of procedures known to those of skill in the art.  
30 These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein.



The presence of polynucleotide sequences encoding CBPP-1 can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes or fragments of polynucleotides encoding CBPP-1. Nucleic acid amplification based assays involve the use of oligonucleotides or oligomers based on the sequences encoding CBPP-1 to detect transformants  
5 containing DNA or RNA encoding CBPP-1.

A variety of protocols for detecting and measuring the expression of CBPP-1, using either polyclonal or monoclonal antibodies specific for the protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing  
10 monoclonal antibodies reactive to two non-interfering epitopes on CBPP-1 is preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton, R. et al. (1990; Serological Methods, a Laboratory Manual, APS Press, St Paul, MN) and Maddox, D.E. et al. (1983; J. Exp. Med. 158:1211-1216).

A wide variety of labels and conjugation techniques are known by those skilled in the art  
15 and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides encoding CBPP-1 include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the sequences encoding CBPP-1, or any fragments thereof may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are  
20 commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits (Pharmacia & Upjohn, (Kalamazoo, MI); Promega (Madison WI); and U.S. Biochemical Corp., Cleveland, OH). Suitable reporter molecules or labels, which may be used for ease of detection, include  
25 radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with nucleotide sequences encoding CBPP-1 may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a transformed cell may be secreted or contained intracellularly depending on  
30 the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode CBPP-1 may be designed to contain signal sequences which direct secretion of CBPP-1 through a prokaryotic or eukaryotic cell membrane. Other constructions may be used to join sequences encoding CBPP-1 to nucleotide sequence

encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, WA). The inclusion of cleavable linker sequences such as those specific for Factor XA or enterokinase (Invitrogen, San Diego, CA) between the purification domain and CBPP-1 may be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing CBPP-1 and a nucleic acid encoding 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification on IMAC (immobilized metal ion affinity chromatography as described in Porath, J. et al. (1992, Prot. Exp. Purif. 3: 263-281) while the enterokinase cleavage site provides a means for purifying CBPP-1 from the fusion protein. A discussion of vectors which contain fusion proteins is provided in Kroll, D.J. et al. (1993; DNA Cell Biol. 12:441-453).

In addition to recombinant production, fragments of CBPP-1 may be produced by direct peptide synthesis using solid-phase techniques Merrifield J. (1963) J. Am. Chem. Soc. 85:2149-2154). Protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Various fragments of CBPP-1 may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

## THERAPEUTICS

Chemical and structural homology exists among CBPP-1 and calcyphosine from dog (GI 877) and human (GI 1359717). In addition, CBPP-1 is expressed in the brain and in fetal tissues. Therefore, CBPP-1 appears to play a role in neurological and developmental disorders, particularly disorders in which CBPP-1 is underexpressed.

Therefore, in one embodiment, CBPP-1 or a fragment or derivative thereof may be administered to a subject to prevent or treat a neurological disorder. Such disorders include, but are not limited to, akathisia, Alzheimer's disease, amnesia, amyotrophic lateral sclerosis, bipolar disorder, catatonia, cerebral neoplasms, dementia, depression, Down's syndrome, tardive dyskinesia, dystonias, epilepsy, Huntington's disease, multiple sclerosis, neurofibromatosis, Parkinson's disease, paranoid psychoses, schizophrenia, and Tourette's disorder.

In another embodiment, a vector capable of expressing CBPP-1, or a fragment or a derivative thereof, may also be administered to a subject to prevent or treat a neurological disorder including, but not limited to, the disorders described above.

In another embodiment, an agonist which modulates the activity of CBPP-1 may also be administered to a subject to prevent or treat a neurological disorder including, but not limited to, the disorders described above.

In another embodiment, CBPP-1 or a fragment or derivative thereof may be administered to a subject to prevent or treat a developmental disorder. The term "developmental disorder" refers to any disorder associated with development or function of a tissue, organ, or system of a subject, i.e., brain, adrenal gland, kidney, skeletal or reproductive system. Such disorders include, but are not limited to, renal tubular acidosis, anemia, Cushing's syndrome, achondroplastic dwarfism, epilepsy, gonadal dysgenesis, hereditary neuropathies such as Charcot-Marie-Tooth disease and neurofibromatosis, hypothyroidism, hydrocephalus, seizure disorders such as Sydenham's chorea and cerebral palsy, spinal bifida, and congenital glaucoma, cataract, or sensorineural hearing loss.

In another embodiment, a vector capable of expressing CBPP-1, or a fragment or a derivative thereof, may also be administered to a subject to prevent or treat a developmental disorder including, but not limited to, the disorders described above.

In another embodiment, an agonist which modulates the activity of CBPP-1 may also be administered to a subject to prevent or treat a developmental disorder including, but not limited to, the disorders described above.

In other embodiments, any of the therapeutic proteins, antagonists, antibodies, agonists, complementary sequences or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents for use in combination therapy may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, one may be able to achieve therapeutic efficacy with lower dosages of each agent, thus reducing the potential for adverse side effects.

Antagonists or inhibitors of CBPP-1 may be produced using methods which are generally known in the art. In particular, purified CBPP-1 may be used to produce antibodies or to screen libraries of pharmaceutical agents to identify those which specifically bind CBPP-1.

Antibodies to CBPP-1 may be generated using methods that are well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, and fragments produced by a Fab expression library. Neutralizing antibodies, (i.e., those which inhibit dimer formation) are especially preferred for therapeutic use.

5 For the production of antibodies, various hosts including goats, rabbits, rats, mice, humans, and others, may be immunized by injection with CBPP-1 or any fragment or oligopeptide thereof which has immunogenic properties. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface active substances  
10 such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and Corynebacterium parvum are especially preferable.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to CBPP-1 have an amino acid sequence consisting of at least five amino acids and more preferably  
15 at least 10 amino acids. It is also preferable that they are identical to a portion of the amino acid sequence of the natural protein, and they may contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of CBPP-1 amino acids may be fused with those of another protein such as keyhole limpet hemocyanin and antibody produced against the chimeric molecule.

20 Monoclonal antibodies to CBPP-1 may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique (Kohler, G. et al. (1975) Nature 256:495-497; Kozbor, D. et al. (1985) J. Immunol. Methods 81:31-42; Cote, R.J. et al. (1983) Proc. Natl. Acad. Sci. 80:2026-2030;  
25 Cole, S.P. et al. (1984) Mol. Cell Biol. 62:109-120).

In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison, S.L. et al. (1984) Proc. Natl. Acad. Sci. 81:6851-6855; Neuberger, M.S. et al. (1984) Nature 312:604-608; Takeda, S. et al. (1985) Nature  
30 314:452-454). Alternatively, techniques described for the production of single chain antibodies may be adapted, using methods known in the art, to produce CBPP-1-specific single chain antibodies. Antibodies with related specificity, but of distinct idiotypic composition, may be

generated by chain shuffling from random combinatorial immunoglobulin libraries (Burton D.R. (1991) Proc. Natl. Acad. Sci. 88:11120-3).

Antibodies may also be produced by inducing in vivo production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature (Orlandi, R. et al. (1989) Proc. Natl. Acad. Sci. 86: 3833-3837; Winter, G. et al. (1991) Nature 349:293-299).

Antibody fragments which contain specific binding sites for CBPP-1 may also be generated. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse, W.D. et al. (1989) Science 254:1275-1281).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between CBPP-1 and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering CBPP-1 epitopes is preferred, but a competitive binding assay may also be employed (Maddox, supra).

In another embodiment of the invention, the polynucleotides encoding CBPP-1, or any fragment or complement thereof, may be used for therapeutic purposes. In one aspect, the complement of the polynucleotide encoding CBPP-1 may be used in situations in which it would be desirable to block the transcription of the mRNA. In particular, cells may be transformed with sequences complementary to polynucleotides encoding CBPP-1. Thus, complementary molecules or fragments may be used to modulate CBPP-1 activity, or to achieve regulation of gene function. Such technology is now well known in the art, and sense or antisense oligonucleotides or larger fragments, can be designed from various locations along the coding or control regions of sequences encoding CBPP-1.

Expression vectors derived from retro viruses, adenovirus, herpes or vaccinia viruses, or from various bacterial plasmids may be used for delivery of nucleotide sequences to the targeted organ, tissue or cell population. Methods which are well known to those skilled in the art can be used to construct vectors which will express nucleic acid sequence which is complementary to

the polynucleotides of the gene encoding CBPP-1. These techniques are described both in Sambrook et al. (supra) and in Ausubel et al. (supra).

Genes encoding CBPP-1 can be turned off by transforming a cell or tissue with expression vectors which express high levels of a polynucleotide or fragment thereof which encodes CBPP-1. Such constructs may be used to introduce untranslatable sense or antisense sequences into a cell. Even in the absence of integration into the DNA, such vectors may continue to transcribe RNA molecules until they are disabled by endogenous nucleases. Transient expression may last for a month or more with a non-replicating vector and even longer if appropriate replication elements are part of the vector system.

As mentioned above, modifications of gene expression can be obtained by designing complementary sequences or antisense molecules (DNA, RNA, or PNA) to the control, 5' or regulatory regions of the gene encoding CBPP-1 (signal sequence, promoters, enhancers, and introns). Oligonucleotides derived from the transcription initiation site, e.g., between positions -10 and +10 from the start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA have been described in the literature (Gee, J.E. et al. (1994) In: Huber, B.E. and B.I. Carr, Molecular and Immunologic Approaches, Futura Publishing Co., Mt. Kisco, NY). The complementary sequence or antisense molecule may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to catalyze the specific cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage.

Examples which may be used include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding CBPP-1.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU, and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

Complementary ribonucleic acid molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding CBPP-1. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, these cDNA constructs that synthesize complementary RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2'O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept is inherent in the production of PNAs and can be extended in all of these molecules by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

Many methods for introducing vectors into cells or tissues are available and equally suitable for use in vivo, in vitro, and ex vivo. For ex vivo therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection, by liposome injections or polycationic amino polymers (Goldman, C.K. et al. (1997) Nature Biotechnology 15:462-66; incorporated herein by reference) may be achieved using methods which are well known in the art.

Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

An additional embodiment of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, for any of the therapeutic effects discussed above. Such pharmaceutical compositions may consist of CBPP-1, antibodies to CBPP-1, mimetics, agonists, antagonists, or inhibitors of CBPP-1. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The

compositions may be administered to a patient alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions utilized in this invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, PA).

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores may be used in conjunction with suitable coatings, such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol.



Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

5           Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be  
10       prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

15           For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

20           The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following: 1-50 mM histidine, 0.1%-2% sucrose, and  
25       2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For administration of CBPP-1, such labeling would include amount, frequency, and method of administration.

30           Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models, usually mice, rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

A therapeutically effective dose refers to that amount of active ingredient, for example CBPP-1 or fragments thereof, antibodies of CBPP-1, agonists, antagonists or inhibitors of CBPP-1, which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or polypeptides will be specific to particular cells, conditions, locations, etc.

**DIAGNOSTICS**

In another embodiment, antibodies which specifically bind CBPP-1 may be used for the diagnosis of conditions or diseases characterized by expression of CBPP-1, or in assays to monitor patients being treated with CBPP-1, agonists, antagonists or inhibitors. The antibodies useful for diagnostic purposes may be prepared in the same manner as those described above for therapeutics. Diagnostic assays for CBPP-1 include methods which utilize the antibody and a label to detect CBPP-1 in human body fluids or extracts of cells or tissues. The antibodies may be used with or without modification, and may be labeled by joining them, either covalently or non-covalently, with a reporter molecule. A wide variety of reporter molecules which are known in the art may be used, several of which are described above.

A variety of protocols including ELISA, RIA, and FACS for measuring CBPP-1 are known in the art and provide a basis for diagnosing altered or abnormal levels of CBPP-1 expression. Normal or standard values for CBPP-1 expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to CBPP-1 under conditions suitable for complex formation. The amount of standard complex formation may be quantified by various methods, but preferably by photometric means. Quantities of CBPP-1 expressed in subject, control and disease, samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

In another embodiment of the invention, the polynucleotides encoding CBPP-1 may be used for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, complementary RNA and DNA molecules, and PNAs. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of CBPP-1 may be correlated with disease. The diagnostic assay may be used to distinguish between absence, presence, and excess expression of CBPP-1, and to monitor regulation of CBPP-1 levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, encoding CBPP-1 or closely related molecules, may be used to identify nucleic acid sequences which encode CBPP-1. The specificity of the probe, whether it is made from a highly specific region, e.g., 10 unique nucleotides in the 5' regulatory region, or a less specific region, e.g., especially in the 3' coding region, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low) will determine whether the probe identifies only naturally occurring sequences encoding CBPP-1, alleles, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from any of the CBPP-1 encoding sequences. The hybridization probes of the subject invention may be DNA or RNA and derived from the nucleotide sequence of SEQ ID NO:2 or from genomic sequence including promoter, enhancer elements, and introns of the naturally occurring CBPP-1.

Means for producing specific hybridization probes for DNAs encoding CBPP-1 include the cloning of nucleic acid sequences encoding CBPP-1 or CBPP-1 derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example, radionuclides such as <sup>32</sup>P or <sup>35</sup>S, or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

Polynucleotide sequences encoding CBPP-1 may be used for the diagnosis of conditions, disorders, or diseases which are associated with expression of CBPP-1. Examples of such conditions or diseases include neurological disorders such as akathisia, Alzheimer's disease, amnesia, amyotrophic lateral sclerosis, bipolar disorder, catatonia, cerebral neoplasms, dementia, depression, Down's syndrome, tardive dyskinesia, dystonias, epilepsy, Huntington's disease, multiple sclerosis, neurofibromatosis, Parkinson's disease, paranoid psychoses, schizophrenia, and Tourette's disorder; and developmental disorders such as renal tubular acidosis, anemia, Cushing's syndrome, achondroplastic dwarfism, epilepsy, gonadal dysgenesis, hereditary neuropathies such as Charcot-Marie-Tooth disease and neurofibromatosis, hypothyroidism, hydrocephalus, seizure disorders such as Sydenham's chorea and cerebral palsy, spinal bifida, and congenital glaucoma, cataract, or sensorineural hearing loss. The polynucleotide sequences encoding CBPP-1 may be used in Southern or northern analysis, dot blot, or other membrane-based technologies; in PCR technologies; or in dipstick, pin, ELISA assays or microarrays utilizing fluids or tissues from patient biopsies to detect altered CBPP-1 expression. Such qualitative or quantitative methods are well known in the art.

In a particular aspect, the nucleotide sequences encoding CBPP-1 may be useful in assays that detect activation or induction of various cancers, particularly those mentioned above. The nucleotide sequences encoding CBPP-1 may be labeled by standard methods, and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated

and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly altered from that of a comparable control sample, the nucleotide sequences have hybridized with nucleotide sequences in the sample, and the presence of altered levels of nucleotide sequences encoding CBPP-1 in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

In order to provide a basis for the diagnosis of disease associated with expression of CBPP-1, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, which encodes CBPP-1, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Additional diagnostic uses for oligonucleotides designed from the sequences encoding CBPP-1 may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced in vitro. Oligomers will preferably consist of two nucleotide sequences, one with sense orientation (5'→3') and another with antisense (3'←5'), employed under optimized conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed

under less stringent conditions for detection and/or quantitation of closely related DNA or RNA sequences.

Methods which may also be used to quantitate the expression of CBPP-1 include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard  
5 curves onto which the experimental results are interpolated (Melby, P.C. et al. (1993) J. Immunol. Methods, 159:235-244; Duplaa, C. et al. (1993) Anal. Biochem. 229-236). The speed of quantitation of multiple samples may be accelerated by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or colorimetric response gives rapid quantitation.

10 In further embodiments, oligonucleotides derived from any of the polynucleotide sequences described herein may be used as targets in microarrays. The microarrays can be used to monitor the expression level of large numbers of genes simultaneously (to produce a transcript image), and to identify genetic variants, mutations and polymorphisms. This information will be useful in determining gene function, understanding the genetic basis of disease, diagnosing  
15 disease, and in developing and monitoring the activity of therapeutic agents (Heller, R. et al. (1997) Proc. Natl. Acad. Sci. 94:2150-55).

In one embodiment, the microarray is prepared and used according to the methods described in PCT application WO95/11995 (Chee et al.), Lockhart, D. J. et al. (1996; Nat. Biotech. 14: 1675-1680) and Schena, M. et al. (1996; Proc. Natl. Acad. Sci. 93: 10614-10619),  
20 all of which are incorporated herein in their entirety by reference.

The microarray is preferably composed of a large number of unique, single-stranded nucleic acid sequences, usually either synthetic antisense oligonucleotides or fragments of cDNAs, fixed to a solid support. The oligonucleotides are preferably about 6-60 nucleotides in length, more preferably 15-30 nucleotides in length, and most preferably about 20-25 nucleotides  
25 in length. For a certain type of microarray, it may be preferable to use oligonucleotides which are only 7-10 nucleotides in length. The microarray may contain oligonucleotides which cover the known 5', or 3', sequence, sequential oligonucleotides which cover the full length sequence; or unique oligonucleotides selected from particular areas along the length of the sequence. Polynucleotides used in the microarray may be oligonucleotides that are specific to a gene or  
30 genes of interest in which at least a fragment of the sequence is known or that are specific to one or more unidentified cDNAs which are common to a particular cell type, developmental or disease state.

In order to produce oligonucleotides to a known sequence for a microarray, the gene of interest is examined using a computer algorithm which starts at the 5' or more preferably at the 3' end of the nucleotide sequence. The algorithm identifies oligomers of defined length that are unique to the gene, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that may interfere with hybridization. In certain situations it may be appropriate to use pairs of oligonucleotides on a microarray. The "pairs" will be identical, except for one nucleotide which preferably is located in the center of the sequence. The second oligonucleotide in the pair (mismatched by one) serves as a control. The number of oligonucleotide pairs may range from two to one million. The oligomers are synthesized at designated areas on a substrate using a light-directed chemical process. The substrate may be paper, nylon or other type of membrane, filter, chip, glass slide or any other suitable solid support.

In another aspect, the oligomers may be synthesized on the surface of the substrate by using a chemical coupling procedure and an ink jet application apparatus, as described in PCT application WO95/251116 (Baldeschweiler et al.) which is incorporated herein in its entirety by reference. In another aspect, a "gridded" array analogous to a dot (or slot) blot may be used to arrange and link cDNA fragments or oligonucleotides to the surface of a substrate using a vacuum system, thermal, UV, mechanical or chemical bonding procedures. An array may be produced by hand or using available devices (slot blot or dot blot apparatus), materials (any suitable solid support), and machines (including robotic instruments) and may contain 8, 24, 96, 384, 1536 or 6144 oligonucleotides, or any other multiple between two and one million which lends itself to the efficient use of commercially available instrumentation.

In order to conduct sample analysis using the microarrays, the RNA or DNA from a biological sample is made into hybridization probes. The mRNA is isolated, and cDNA is produced and used as a template to make antisense RNA (aRNA). The aRNA is amplified in the presence of fluorescent nucleotides, and labeled probes are incubated with the microarray so that the probe sequences hybridize to complementary oligonucleotides of the microarray. Incubation conditions are adjusted so that hybridization occurs with precise complementary matches or with various degrees of less complementarity. After removal of nonhybridized probes, a scanner is used to determine the levels and patterns of fluorescence. The scanned images are examined to determine degree of complementarity and the relative abundance of each oligonucleotide sequence on the microarray. The biological samples may be obtained from any bodily fluids (such as blood, urine, saliva, phlegm, gastric juices, etc.), cultured cells, biopsies, or other tissue

preparations. A detection system may be used to measure the absence, presence, and amount of hybridization for all of the distinct sequences simultaneously. This data may be used for large scale correlation studies on the sequences, mutations, variants, or polymorphisms among samples.

5 In another embodiment of the invention, the nucleic acid sequences which encode CBPP-1 may also be used to generate hybridization probes which are useful for mapping the naturally occurring genomic sequence. The sequences may be mapped to a particular chromosome, to a specific region of a chromosome or to artificial chromosome constructions, such as human artificial chromosomes (HACs), yeast artificial chromosomes (YACs), bacterial artificial  
10 chromosomes (BACs), bacterial P1 constructions or single chromosome cDNA libraries as reviewed in Price, C.M. (1993) *Blood Rev.* 7:127-134, and Trask, B.J. (1991) *Trends Genet.* 7:149-154.

Fluorescent in situ hybridization (FISH as described in Verma et al. (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York, NY) may be  
15 correlated with other physical chromosome mapping techniques and genetic map data. Examples of genetic map data can be found in various scientific journals or at Online Mendelian Inheritance in Man (OMIM). Correlation between the location of the gene encoding CBPP-1 on a physical chromosomal map and a specific disease, or predisposition to a specific disease, may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the  
20 subject invention may be used to detect differences in gene sequences between normal, carrier, or affected individuals.

In situ hybridization of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers may be used for extending genetic maps. Often the placement of a gene on the chromosome of another mammalian species, such as  
25 mouse, may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once the disease or syndrome has been crudely localized by genetic linkage to a particular genomic region, for  
30 example, AT to 11q22-23 (Gatti, R.A. et al. (1988) *Nature* 336:577-580), any sequences mapping to that area may represent associated or regulatory genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. among normal, carrier, or affected individuals.



In another embodiment of the invention, CBPP-1, its catalytic or immunogenic fragments or oligopeptides thereof, can be used for screening libraries of compounds in any of a variety of drug screening techniques. The fragment employed in such screening may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The formation of binding complexes, between CBPP-1 and the agent being tested, may be measured.

Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the protein of interest as described in published PCT application WO84/03564. In this method, as applied to CBPP-1 large numbers of different small test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The test compounds are reacted with CBPP-1, or fragments thereof, and washed. Bound CBPP-1 is then detected by methods well known in the art. Purified CBPP-1 can also be coated directly onto plates for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

In another embodiment, one may use competitive drug screening assays in which neutralizing antibodies capable of binding CBPP-1 specifically compete with a test compound for binding CBPP-1. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants with CBPP-1.

In additional embodiments, the nucleotide sequences which encode CBPP-1 may be used in any molecular biology techniques that have yet to be developed, provided the new techniques rely on properties of nucleotide sequences that are currently known, including, but not limited to, such properties as the triplet genetic code and specific base pair interactions.

The examples below are provided to illustrate the subject invention and are not included for the purpose of limiting the invention.

## EXAMPLES

### I LUNGFET03 cDNA Library Construction

The LUNGFET03 cDNA library was constructed from fetal lung tissue obtained from an anencephalic female Caucasian fetus who died at 20 weeks gestation (specimen #RU95-10-0739; International Institute of Advanced Medicine, Exton, PA). The Mother's medical history included seven days of treatment with erythromycin for bronchitis during the first trimester.

The frozen tissue was homogenized and lysed using a Brinkmann Homogenizer Polytron PT-3000 (Brinkmann Instruments, Westbury, NJ) in guanidinium isothiocyanate solution. The

lysate was centrifuged over a 5.7 M CsCl cushion using an Beckman SW28 rotor in a Beckman L8-70M Ultracentrifuge (Beckman Instruments) for 18 hours at 25,000 rpm at ambient temperature. The RNA was extracted with acid phenol pH 4.7, precipitated using 0.3 M sodium acetate and 2.5 volumes of ethanol, resuspended in RNase-free water, and DNase treated at 37°C. Extraction and precipitation were repeated as before. The mRNA was then isolated using the Qiagen Oligotex kit (QIAGEN, Inc., Chatsworth, CA) and used to construct the cDNA library.

The mRNA was handled according to the recommended protocols in the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (Cat. #18248-013, Gibco/BRL, Gaithersburg, MD). cDNAs were fractionated on a Sepharose CL4B column (Cat. #275105-01, Pharmacia), and those cDNAs exceeding 400 bp were ligated into PSPO I. The plasmid PSPO I was subsequently transformed into DH5a<sup>TM</sup> competent cells (Cat. #18258-012, Gibco/BRL).

## **II Isolation and Sequencing of cDNA Clones**

Plasmid DNA was released from the cells and purified using the REAL Prep 96 Plasmid Kit (Catalog #26173, QIAGEN, Inc.). This kit enabled the simultaneous purification of 96 samples in a 96-well block using multi-channel reagent dispensers. The recommended protocol was employed except for the following changes: 1) the bacteria were cultured in 1 ml of sterile Terrific Broth (Catalog #22711, Gibco/BRL) with carbenicillin at 25 mg/L and glycerol at 0.4%; 2) after inoculation, the cultures were incubated for 19 hours and at the end of incubation, the cells were lysed with 0.3 ml of lysis buffer; and 3) following isopropanol precipitation, the plasmid DNA pellet was resuspended in 0.1 ml of distilled water. After the last step in the protocol, samples were transferred to a 96-well block for storage at 4° C.

The cDNAs were sequenced by the method of Sanger et al. (1975, J. Mol. Biol. 94:441f), using a Hamilton Micro Lab 2200 (Hamilton, Reno, NV) in combination with Peltier Thermal Cyclers (PTC200 from MJ Research, Watertown, MA) and Applied Biosystems 377 DNA Sequencing Systems.

## **III Homology Searching of cDNA Clones and Their Deduced Proteins**

The nucleotide sequences of the Sequence Listing or amino acid sequences deduced from them were used as query sequences against databases such as GenBank, SwissProt, BLOCKS, and Pima II. These databases which contain previously identified and annotated sequences were

searched for regions of homology (similarity) using BLAST, which stands for Basic Local Alignment Search Tool (Altschul, S.F. (1993) J. Mol. Evol. 36:290-300; Altschul et al. (1990) J. Mol. Biol. 215:403-410).

BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs which may be of prokaryotic (bacterial) or eukaryotic (animal, fungal or plant) origin. Other algorithms such as the one described in Smith RF and TF Smith (1992; Protein Engineering 5:35-51), incorporated herein by reference, can be used when dealing with primary sequence patterns and secondary structure gap penalties. As disclosed in this application, the sequences have lengths of at least 49 nucleotides, and no more than 12% uncalled bases (where N is recorded rather than A, C, G, or T).

The BLAST approach, as detailed in Karlin, S. and S.F. Atschul (1993; Proc. Nat. Acad. Sci. 90:5873-7) and incorporated herein by reference, searches for matches between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. In this application, threshold was set at  $10^{-25}$  for nucleotides and  $10^{-14}$  for peptides.

Incyte nucleotide sequences were searched against the GenBank databases for primate (pri), rodent (rod), and mammalian sequences (mam), and deduced amino acid sequences from the same clones are searched against GenBank functional protein databases, mammalian (mamp), vertebrate (vrtp) and eukaryote (eukp), for homology. The relevant database for a particular match were reported as a Gxxxx±p (where xxx is pri, rod, etc and if present, p = peptide).

#### IV Northern Analysis

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which RNAs from a particular cell type or tissue have been bound (Sambrook et al., supra).

Analogous computer techniques using BLAST (Altschul, S.F. 1993 and 1990, supra) are used to search for identical or related molecules in nucleotide databases such as GenBank or the LIFESEQ™ database (Incyte Pharmaceuticals). This analysis is much faster than multiple, membrane-based hybridizations. In addition, the sensitivity of the computer search can be modified to determine whether any particular match is categorized as exact or homologous.

The basis of the search is the product score which is defined as:

$$\frac{\% \text{ sequence identity} \times \% \text{ maximum BLAST score}}{2}$$

The product score takes into account both the degree of similarity between two sequences and the length of the sequence match. For example, with a product score of 40, the match will be exact within a 1-2% error; and at 70, the match will be exact. Homologous molecules are usually identified by selecting those which show product scores between 15 and 40, although lower scores may identify related molecules.

The results of northern analysis are reported as a list of libraries in which the transcript encoding CBPP-1 occurs. Abundance and percent abundance are also reported. Abundance directly reflects the number of times a particular transcript is represented in a cDNA library, and percent abundance is abundance divided by the total number of sequences examined in the cDNA library.

## V Extension of CBPP-1 Encoding Polynucleotides

The nucleic acid sequence of the Incyte Clone 1850226 was used to design oligonucleotide primers for extending a partial nucleotide sequence to full length. One primer was synthesized to initiate extension in the antisense direction, and the other was synthesized to extend sequence in the sense direction. Primers were used to facilitate the extension of the known sequence "outward" generating amplicons containing new, unknown nucleotide sequence for the region of interest. The initial primers were designed from the cDNA using OLIGO 4.06 (National Biosciences), or another appropriate program, to be about 22 to about 30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures of about 68° to about 72° C. Any stretch of nucleotides which would result in hairpin structures and primer-primer dimerizations was avoided.

Selected human cDNA libraries (Gibco/BRL) were used to extend the sequence. If more than one extension is necessary or desired, additional sets of primers are designed to further extend the known region.

High fidelity amplification was obtained by following the instructions for the XL-PCR kit (Perkin Elmer) and thoroughly mixing the enzyme and reaction mix. Beginning with 40 pmol of each primer and the recommended concentrations of all other components of the kit, PCR was performed using the Peltier Thermal Cycler (PTC200; M.J. Research, Watertown, MA) and the following parameters:

Step 1	94° C for 1 min (initial denaturation)
Step 2	65° C for 1 min
Step 3	68° C for 6 min

40

	Step 4	94° C for 15 sec
	Step 5	65° C for 1 min
	Step 6	68° C for 7 min
	Step 7	Repeat step 4-6 for 15 additional cycles
5	Step 8	94° C for 15 sec
	Step 9	65° C for 1 min
	Step 10	68° C for 7:15 min
	Step 11	Repeat step 8-10 for 12 cycles
	Step 12	72° C for 8 min
10	Step 13	4° C (and holding)

A 5-10  $\mu$ l aliquot of the reaction mixture was analyzed by electrophoresis on a low concentration (about 0.6-0.8%) agarose mini-gel to determine which reactions were successful in extending the sequence. Bands thought to contain the largest products were excised from the gel, purified using QIA QUICK™ (QIAGEN Inc., Chatsworth, CA), and trimmed of overhangs using Klenow enzyme to facilitate religation and cloning.

After ethanol precipitation, the products were redissolved in 13  $\mu$ l of ligation buffer, 1  $\mu$ l T4-DNA ligase (15 units) and 1  $\mu$ l T4 polynucleotide kinase were added, and the mixture was incubated at room temperature for 2-3 hours or overnight at 16° C. Competent *E. coli* cells (in 40  $\mu$ l of appropriate media) were transformed with 3  $\mu$ l of ligation mixture and cultured in 80  $\mu$ l of SOC medium (Sambrook et al., supra). After incubation for one hour at 37° C, the *E. coli* mixture was plated on Luria Bertani (LB)-agar (Sambrook et al., supra) containing 2x Carb. The following day, several colonies were randomly picked from each plate and cultured in 150  $\mu$ l of liquid LB/2x Carb medium placed in an individual well of an appropriate, commercially-available, sterile 96-well microtiter plate. The following day, 5  $\mu$ l of each overnight culture was transferred into a non-sterile 96-well plate and after dilution 1:10 with water, 5  $\mu$ l of each sample was transferred into a PCR array.

For PCR amplification, 18  $\mu$ l of concentrated PCR reaction mix (3.3x) containing 4 units of rTth DNA polymerase, a vector primer, and one or both of the gene specific primers used for the extension reaction were added to each well. Amplification was performed using the following conditions:

	Step 1	94° C for 60 sec
	Step 2	94° C for 20 sec
	Step 3	55° C for 30 sec
35	Step 4	72° C for 90 sec
	Step 5	Repeat steps 2-4 for an additional 29 cycles
	Step 6	72° C for 180 sec
	Step 7	4° C (and holding)

Aliquots of the PCR reactions were run on agarose gels together with molecular weight markers. The sizes of the PCR products were compared to the original partial cDNAs, and appropriate clones were selected, ligated into plasmid, and sequenced.

In like manner, the nucleotide sequence of SEQ ID NO:2 is used to obtain 5' regulatory sequences using the procedure above, oligonucleotides designed for 5' extension, and an appropriate genomic library.

## VI Labeling and Use of Individual Hybridization Probes

Hybridization probes derived from SEQ ID NO:2 are employed to screen cDNAs, genomic DNAs, or mRNAs. Although the labeling of oligonucleotides, consisting of about 20 base-pairs, is specifically described, essentially the same procedure is used with larger nucleotide fragments. Oligonucleotides are designed using state-of-the-art software such as OLIGO 4.06 (National Biosciences), labeled by combining 50 pmol of each oligomer and 250  $\mu$ Ci of [ $\gamma$ - $^{32}$ P] adenosine triphosphate (Amersham) and T4 polynucleotide kinase (DU PONT NEN<sup>®</sup>, Boston, MA). The labeled oligonucleotides are substantially purified with Sephadex G-25 superfine resin column (Pharmacia & Upjohn). A aliquot containing  $10^7$  counts per minute of the labeled probe is used in a typical membrane-based hybridization analysis of human genomic DNA digested with one of the following endonucleases (Ase I, Bgl II, Eco RI, Pst I, Xba I, or Pvu II; DU PONT NEN<sup>®</sup>).

The DNA from each digest is fractionated on a 0.7 percent agarose gel and transferred to nylon membranes (Nytran Plus, Schleicher & Schuell, Durham, NH). Hybridization is carried out for 16 hours at 40°C. To remove nonspecific signals, blots are sequentially washed at room temperature under increasingly stringent conditions up to 0.1 x saline sodium citrate and 0.5% sodium dodecyl sulfate. After XOMAT AR<sup>™</sup> film (Kodak, Rochester, NY) is exposed to the blots in a Phosphoimager cassette (Molecular Dynamics, Sunnyvale, CA) for several hours, hybridization patterns are compared visually.

## VII Microarrays

To produce oligonucleotides for a microarray, the nucleotide sequence described herein is examined using a computer algorithm which starts at the 3' end of the nucleotide sequence. The algorithm identifies oligomers of defined length that are unique to the gene, have a GC content within a range suitable for hybridization, and lack predicted secondary structure that would interfere with hybridization. The algorithm identifies 20 sequence-specific

oligonucleotides of 20 nucleotides in length (20-mers). A matched set of oligonucleotides is created in which one nucleotide in the center of each sequence is altered. This process is repeated for each gene in the microarray, and double sets of twenty 20 mers are synthesized and arranged on the surface of the silicon chip using a light-directed chemical process (Chee, M. et al.,  
5 PCT/WO95/11995, incorporated herein by reference).

In the alternative, a chemical coupling procedure and an ink jet device are used to synthesize oligomers on the surface of a substrate (Baldeschweiler, J.D. et al., PCT/WO95/25116, incorporated herein by reference). In another alternative, a "gridded" array analogous to a dot (or slot) blot is used to arrange and link cDNA fragments or oligonucleotides  
10 to the surface of a substrate using a vacuum system, thermal, UV, mechanical or chemical bonding procedures. An array may be produced by hand or using available materials and machines and contain grids of 8 dots, 24 dots, 96 dots, 384 dots, 1536 dots or 6144 dots. After hybridization, the microarray is washed to remove nonhybridized probes, and a scanner is used to determine the levels and patterns of fluorescence. The scanned images are examined to  
15 determine degree of complementarity and the relative abundance of each oligonucleotide sequence on the micro-array.

### VIII Complementary Polynucleotides

Sequence complementary to the CBPP-1-encoding sequence, or any part thereof, is used  
20 to decrease or inhibit expression of naturally occurring CBPP-1. Although use of oligonucleotides comprising from about 15 to about 30 base-pairs is described, essentially the same procedure is used with smaller or larger sequence fragments. Appropriate oligonucleotides are designed using Oligo 4.06 software and the coding sequence of CBPP-1, SEQ ID NO:1. To inhibit transcription, a complementary oligonucleotide is designed from the most unique 5'  
25 sequence and used to prevent promoter binding to the coding sequence. To inhibit translation, a complementary oligonucleotide is designed to prevent ribosomal binding to the CBPP-1-encoding transcript.

### IX Expression of CBPP-1

30 Expression of CBPP-1 is accomplished by subcloning the cDNAs into appropriate vectors and transforming the vectors into host cells. In this case, the cloning vector is also used to express CBPP-1 in *E. coli*. Upstream of the cloning site, this vector contains a promoter for  $\beta$ -galactosidase, followed by sequence containing the amino-terminal Met, and the subsequent

seven residues of  $\beta$ -galactosidase. Immediately following these eight residues is a bacteriophage promoter useful for transcription and a linker containing a number of unique restriction sites.

Induction of an isolated, transformed bacterial strain with IPTG using standard methods produces a fusion protein which consists of the first eight residues of  $\beta$ -galactosidase, about 5 to 15 residues of linker, and the full length protein. The signal residues direct the secretion of CBPP-1 into the bacterial growth media which can be used directly in the following assay for activity.

#### **X Demonstration of CBPP-1 Activity**

Calcium-binding activity of CBPP-1 may be demonstrated by incubating purified CBPP-1 in a buffer together with radioactive calcium ( $^{45}\text{Ca}$ ). An aliquot of the incubation is then subjected to gel electrophoresis to separate the free  $^{45}\text{Ca}$  from  $^{45}\text{Ca}$ -bound CBPP-1. The  $^{45}\text{Ca}$ -bound CBPP-1 is detected by autoradiography and counted in a radioisotope counter. The amount of radioactivity recovered is proportional to the amount of CBPP-1 in the incubation.

#### **XI Production of CBPP-1 Specific Antibodies**

CBPP-1 that is substantially purified using PAGE electrophoresis (Sambrook, supra), or other purification techniques, is used to immunize rabbits and to produce antibodies using standard protocols. The amino acid sequence deduced from SEQ ID NO:2 is analyzed using DNASTAR software (DNASTAR Inc) to determine regions of high immunogenicity and a corresponding oligopeptide is synthesized and used to raise antibodies by means known to those of skill in the art. Selection of appropriate epitopes, such as those near the C-terminus or in hydrophilic regions, is described by Ausubel et al. (supra), and others.

Typically, the oligopeptides are 15 residues in length, synthesized using an Applied Biosystems Peptide Synthesizer Model 431A using fmoc-chemistry, and coupled to keyhole limpet hemocyanin (KLH, Sigma, St. Louis, MO) by reaction with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Ausubel et al., supra). Rabbits are immunized with the oligopeptide-KLH complex in complete Freund's adjuvant. The resulting antisera are tested for antipeptide activity, for example, by binding the peptide to plastic, blocking with 1% BSA, reacting with rabbit antisera, washing, and reacting with radio iodinated, goat anti-rabbit IgG.



**XII Purification of Naturally Occurring CBPP-1 Using Specific Antibodies**

Naturally occurring or recombinant CBPP-1 is substantially purified by immunoaffinity chromatography using antibodies specific for CBPP-1. An immunoaffinity column is constructed by covalently coupling CBPP-1 antibody to an activated chromatographic resin, such as  
5 CNBr-activated Sepharose (Pharmacia & Upjohn). After the coupling, the resin is blocked and washed according to the manufacturer's instructions.

Media containing CBPP-1 is passed over the immunoaffinity column, and the column is washed under conditions that allow the preferential absorbance of CBPP-1 (e.g., high ionic strength buffers in the presence of detergent). The column is eluted under conditions that disrupt  
10 antibody/CBPP-1 binding (eg, a buffer of pH 2-3 or a high concentration of a chaotrope, such as urea or thiocyanate ion), and CBPP-1 is collected.

**XIII Identification of Molecules Which Interact with CBPP-1**

CBPP-1 or biologically active fragments thereof are labeled with <sup>125</sup>I Bolton-Hunter  
15 reagent (Bolton et al. (1973) Biochem. J. 133: 529). Candidate molecules previously arrayed in the wells of a multi-well plate are incubated with the labeled CBPP-1, washed and any wells with labeled CBPP-1 complex are assayed. Data obtained using different concentrations of CBPP-1 are used to calculate values for the number, affinity, and association of CBPP-1 with the candidate molecules.

20 All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited  
25 to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

## SEQUENCE LISTING

- (1) GENERAL INFORMATION
- (i) APPLICANT: INCYTE PHARMACEUTICALS, INC.
- (ii) TITLE OF THE INVENTION: CALCIUM-BINDING PHOSPHOPROTEIN
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Incyte Pharmaceuticals, Inc.
- (B) STREET: 3174 Porter Drive
- (C) CITY: Palo Alto
- (D) STATE: CA
- (E) COUNTRY: USA
- (F) ZIP: 94304
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette
- (B) COMPUTER: IBM Compatible
- (C) OPERATING SYSTEM: DOS
- (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
- (A) PCT APPLICATION NUMBER: To Be Assigned
- (B) FILING DATE: Filed Herewith
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 08/884,682
- (B) FILING DATE: June 27, 1997
- (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Billings, Lucy J.
- (B) REGISTRATION NUMBER: 36,749
- (C) REFERENCE/DOCKET NUMBER: PF-0330 PCT
- (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 650-855-0555
- (B) TELEFAX: 650-845-4166
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 208 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (vii) IMMEDIATE SOURCE:
- (A) LIBRARY: LUNGFET03
- (B) CLONE: 1850226

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Met Ala Gly Thr Ala Arg His Asp Arg Glu Met Ala Ile Gln Ala Lys
 1      5      10      15
Lys Lys Leu Thr Ala Thr Asp Pro Ile Glu Arg Leu Arg Leu Gln
 20      25      30
Cys Leu Ala Arg Gly Ser Ala Gly Ile Lys Gly Leu Gly Arg Val Phe
 35      40      45
Arg Ile Met Asp Asp Asp Asn Asn Arg Thr Leu Asp Phe Lys Glu Phe
 50      55      60
Met Lys Gly Leu Asn Asp Tyr Ala Val Val Met Glu Lys Glu Glu Val
 65      70      75      80
Glu Glu Leu Phe Arg Arg Phe Asp Lys Asp Gly Asn Gly Thr Ile Asp
 85      90      95
Phe Asn Glu Phe Leu Leu Thr Leu Arg Pro Pro Met Ser Arg Ala Arg
100      105      110
Lys Glu Val Ile Met Gln Ala Phe Arg Lys Leu Asp Lys Thr Gly Asp
115      120      125
Gly Val Ile Thr Ile Glu Asp Leu Arg Glu Val Tyr Asn Ala Lys His
130      135      140
His Pro Lys Tyr Gln Asn Gly Glu Trp Ser Glu Glu Gln Val Phe Arg
145      150      155      160
Lys Phe Leu Asp Asn Phe Asp Ser Pro Tyr Asp Lys Asp Gly Leu Val
165      170      175
Thr Pro Glu Glu Phe Met Asn Tyr Tyr Ala Gly Val Ser Ala Ser Ile
180      185      190
Asp Thr Asp Val Tyr Phe Ile Ile Met Met Arg Thr Ala Trp Lys Leu
195      200      205

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 839 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: LUNGFET03
- (B) CLONE: 1850226

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

TTTCAGTTCG AAGCAACTGG TGGCAAAAGG TTAGCATTTA AGATGGCAGG GACAGCGCGC 60
CATGACCGAG AGATGGCGAT CCAGGCCAAG AAAAAGCTCA CCACGGCCAC CGACCCCAT 120
GAAAGACTCC GACTGCAGTG CCTGGCCAGG GGCTCTGCTG GGATCAAAGG ACTTGGCAGA 180
GTGTTTAGAA TTATGGATGA CGATAATAAT CGAACCCTTG ATTTTAAAGA ATTTATGAAA 240
GGGTAAATG ATTATGCTGT GGTCATGGAA AAAGAAGAGG TGGAAGAACT TTTCCGGAGG 300
TTTGATAAAG ATGGAAATGG AACAATAGAC TTCAATGAAT TTCTTCTCAC ATTAAGACCT 360
CCAATGTCCA GAGCCAGAAA AGAGGTAATC ATGCAAGCTT TTAGAAAGTT AGACAAGACT 420
GGAGATGGTG TTATAACAAT CGAAGACCTT CGTGAAGTAT ATAATGCAAA ACACCACCCA 480
AAGTACCAGA ATGGGGAATG GAGTGAGGAA CAAGTATTTA GGAAATTTCT GGATAACTTT 540
GATTCACCC TATGACAAAGA TGGATTGGTG ACCCCTGAGG AGTTCATGAA CTACTATGCA 600
GGTGTGAGCG CATCCATTGA CACTGATGTG TACTTCATCA TCATGATGAG AACCGCCTGG 660
AAGCTTTAAG CACATGACCT GGGGACCAGG CCCTGGGACA GCCATGTGGC TCCAAATGAC 720
TAAATGTCAG CTCAAAAACC AGAATCGTAT TTGATTTTAC ACTCATCCTA ATGTTTTTTT 780
CTGTGTCAAA ATATTGCATT TTCTGGGGCC AAAAACAGG CAGAAATAAA AGCATTGAT 839

```

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 189 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

(A) LIBRARY: GenBank

(B) CLONE: 877

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

Met Asp Ala Val Asp Ala Thr Val Glu Lys Leu Arg Ala Gln Cys Leu
 1          5          10          15
Ser Arg Gly Ala Leu Gly Ile Gln Gly Leu Ala Arg Phe Phe Arg Arg
 20          25          30
Leu Asp Arg Asp Arg Ser Arg Ser Leu Asp Ser Arg Glu Leu Gln Arg
 35          40          45
Gly Leu Ala Glu Leu Gly Leu Val Leu Asp Thr Ala Glu Ala Glu Gly
 50          55          60
Val Cys Arg Arg Trp Asp Arg Asp Gly Ser Gly Thr Leu Asp Leu Glu
 65          70          75          80
Glu Phe Leu Arg Ala Leu Arg Pro Pro Met Ser Gln Ala Arg Glu Ala
 85          90          95
Val Ile Ala Ala Ala Phe Ala Lys Leu Asp Arg Ser Gly Asp Gly Val
100          105          110
Val Thr Val Asp Asp Leu Arg Gly Val Tyr Ser Gly Arg Thr His Pro
115          120          125
Lys Val Gln Ser Gly Glu Trp Thr Glu Glu Glu Val Leu Arg Arg Phe
130          135          140
Leu Asp Asn Phe Asp Ser Ser Glu Lys Asp Gly Gln Val Thr Leu Ala
145          150          155          160
Glu Phe Gln Asp Tyr Tyr Ser Gly Val Ser Ala Ser Met Asp Thr Asp
165          170          175
Glu Glu Phe Val Ala Met Met Thr Ser Ala Trp Gln Leu
180          185

```

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 189 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

(A) LIBRARY: GenBank

(B) CLONE: 1359717

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Asp Ala Val Asp Ala Thr Met Glu Lys Leu Arg Ala Gln Cys Leu
 1          5          10          15
Ser Arg Gly Ala Ser Gly Ile Gln Gly Leu Ala Arg Phe Phe Arg Gln
 20          25          30
Leu Asp Arg Asp Gly Ser Arg Ser Leu Asp Ala Asp Glu Phe Arg Gln
 35          40          45
Gly Leu Ala Lys Leu Gly Leu Val Leu Asp Gln Ala Glu Ala Glu Gly
 50          55          60
Val Cys Arg Lys Trp Asp Arg Asn Gly Ser Gly Thr Leu Asp Leu Glu
 65          70          75          80
Glu Phe Leu Arg Ala Leu Arg Pro Pro Met Ser Gln Ala Arg Glu Ala
 85          90          95
Val Ile Ala Ala Ala Phe Ala Lys Leu Asp Arg Ser Gly Asp Gly Val
100          105          110
Val Thr Val Asp Asp Leu Arg Gly Val Tyr Ser Gly Arg Ala His Pro
115          120          125
Lys Val Arg Ser Gly Glu Trp Thr Glu Asp Glu Val Leu Arg Arg Phe
130          135          140
Leu Asp Asn Phe Asp Ser Ser Glu Lys Asp Gly Gln Val Thr Leu Ala
145          150          155          160

```

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Glu	Phe	Gln	Asp	Tyr	Tyr	Ser	Gly	Val	Ser	Ala	Ser	Met	Asn	Thr	Asp
				165					170					175	
Glu	Glu	Phe	Val	Ala	Met	Met	Thr	Ser	Ala	Trp	Gln	Leu			
			180					185							

What is claimed is:

1. A substantially purified calcium-binding phosphoprotein comprising the amino acid sequence of SEQ ID NO:1 or fragments thereof.

5

2. An isolated and purified polynucleotide sequence encoding the calcium-binding phosphoprotein of claim 1 or fragments or variants of said polynucleotide sequence.

3. A composition comprising the polynucleotide sequence of claim 2.

10

4. A polynucleotide sequence which hybridizes to the polynucleotide sequence of claim 2.

5. A polynucleotide sequence which is complementary to the polynucleotide sequence of claim 2 or fragments or variants thereof.

15

6. An isolated and purified polynucleotide sequence comprising SEQ ID NO:2 or fragments or variants thereof.

20

7. A composition comprising the polynucleotide sequence of claim 6.

8. A polynucleotide sequence which is complementary to the polynucleotide sequence of claim 6.

25

9. An expression vector containing at least a fragment of the polynucleotide sequence of claim 2.

10. A host cell containing the vector of claim 9.

30 11. A method for producing a polypeptide comprising the amino acid sequence of SEQ ID NO:1, or a fragment thereof, the method comprising the steps of:

a) culturing the host cell of claim 10 under conditions suitable for the expression of the polypeptide; and

50

- b) recovering the polypeptide from the host cell culture.

12. A pharmaceutical composition comprising a substantially purified calcium-binding phosphoprotein having the amino acid sequence of SEQ ID NO:1 in conjunction  
5 with a suitable pharmaceutical carrier.

13. A purified antibody of the polypeptide of claim 1.

14. A purified agonist of the polypeptide of claim 1.

15. A purified antagonist of the polypeptide of claim 1.

16. A method for treating a neurological disorder comprising administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim  
15 12.

17. A method for treating a developmental disorder comprising administering to a subject in need of such treatment an effective amount of the pharmaceutical composition of claim  
12.

18. A method for detecting a polynucleotide which encodes a calcium-binding phosphoprotein in a biological sample comprising the steps of:

a) hybridizing the polynucleotide of claim 5 to nucleic acid material of a biological sample, thereby forming a hybridization complex; and

b) detecting said hybridization complex, wherein the presence of said complex correlates with the presence of a polynucleotide encoding calcium-binding phosphoprotein in said biological sample.

19. The method of claim 18 wherein the nucleic acid material is amplified by the  
30 polymerase chain reaction prior to hybridization.

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9	18	27	36	45	54
TTT CAG TTC GAA GCA ACT GGT GGC AAA AGG TTA GCA TTT AAG ATG GCA GGG ACA					
				M A G T	
63	72	81	90	99	108
GGC CGC CAT GAC CGA GAG ATG GCG ATC CAG GCC AAG AAA AAG CTC ACC ACG GCC					
A R H D R E M A I Q A K K K L T T A					
117	126	135	144	153	162
ACC GAC CCC ATT GAA AGA CTC CGA CTG CAG TGC CTG GCC AGG GGC TCT GCT GGG					
T D P I E R L R L Q C L A R G S A G					
171	180	189	198	207	216
ATC AAA GGA CTT GGC AGA GTG TTT AGA ATT ATG GAT GAC GAT AAT AAT CGA ACC					
I K G L G R V F R I M D D D N N R T					
225	234	243	252	261	270
CTT GAT TTT AAA GAA TTT ATG AAA GGG TTA AAT GAT TAT GCT GTG ATG GAA					
L D F K E F M K G L N D Y A V V M E					
279	288	297	306	315	324
AAA GAA GAG GTG GAA GAA CTT TTC CGG AGG TTT GAT AAA GAT GGA AAT GGA ACA					
K E E V E E L F R R R F D K D G N G T					

FIGURE 1A



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333	ATA GAC TTC	342	AAT GAA TTT	351	ACA TTA	360	CCT AGA	369	CCA ATG	378	TCC AGA
I D F	N E	F L	L L	T L	R L	P P	M S	R A	R		
387	AAA GAG GTA	396	ATC ATG CAA	405	AGA AAG	414	GAC TTA	423	ACT GGA	432	GAT GGT
K E V	I M Q	A A	F A	R R	K L	D D	T G	D G	V		
441	ATA ACA ATC	450	GAA GAC CTT	459	GTA GAA	468	GCA AAT	477	CAC CAC	486	CCA AAG
I T I	E D L	R R	E E	V V	Y N	A A	K H	P K	Y		
495	CAG AAT GGG	504	GAA TGG AGT	513	CAA CAA	522	AGG TTT	531	CTG GAT	540	AAC TTT
Q N G	E W S	S E	E E	Q Q	V V	R R	F F	L D	N F		
549	GAT TCA CCC	558	TAT GAC AAA	567	TTG GGA	576	ACC CCT	585	GAG TTC	594	ATG AAC
D S P	Y D K	D D	G G	L L	V V	T P	E E	F M	N Y		
603	TAT GCA GGT	612	GTG AGC GCA	621	GAC ATT	630	GTG TAC	639	ATC ATC	648	ATG ATG
Y A G	V S A	S S	I I	D D	T D	V V	Y Y	I I	M M		

FIGURE 1B

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657	AGA ACC GCC TGG AAG CTT TAA GCA CAT GAC CTG GGG ACC AGG CCC TGG GAC AGC	675	684	693	702
	R T A W K L				
711	CAT GTG GCT CCA AAT GAC TAA ATG TCA GCT CAA AAA CCA GAA TCG TAT TTG ATT	720	729	738	747
765	TCA CAC TCA TCC TAA TGT TTT TTT CTG TGT CAA AAT ATT GCA TTT TCT GGG GCC	774	783	792	801
819	AAA AAA CAG GCA GAA ATA AAA GCA TTG AT	828	837		

FIGURE 1C

## FIGURE 2

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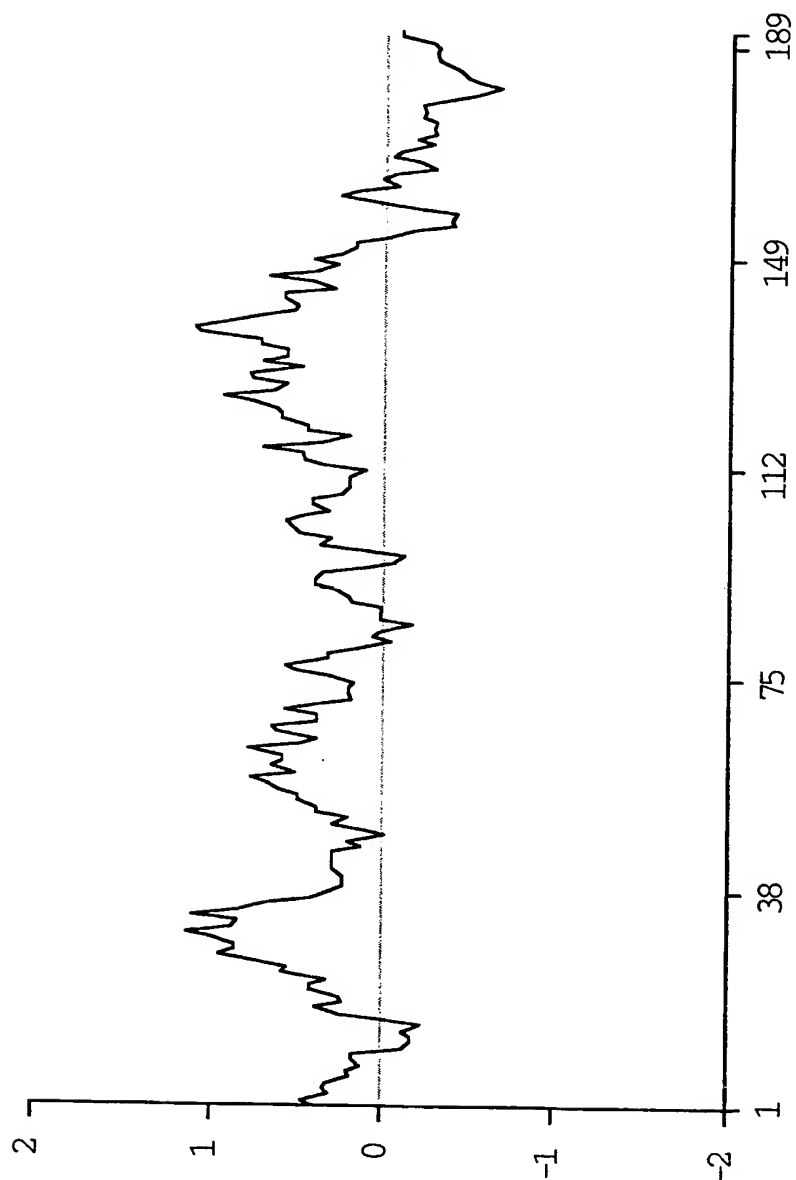


FIGURE 3A

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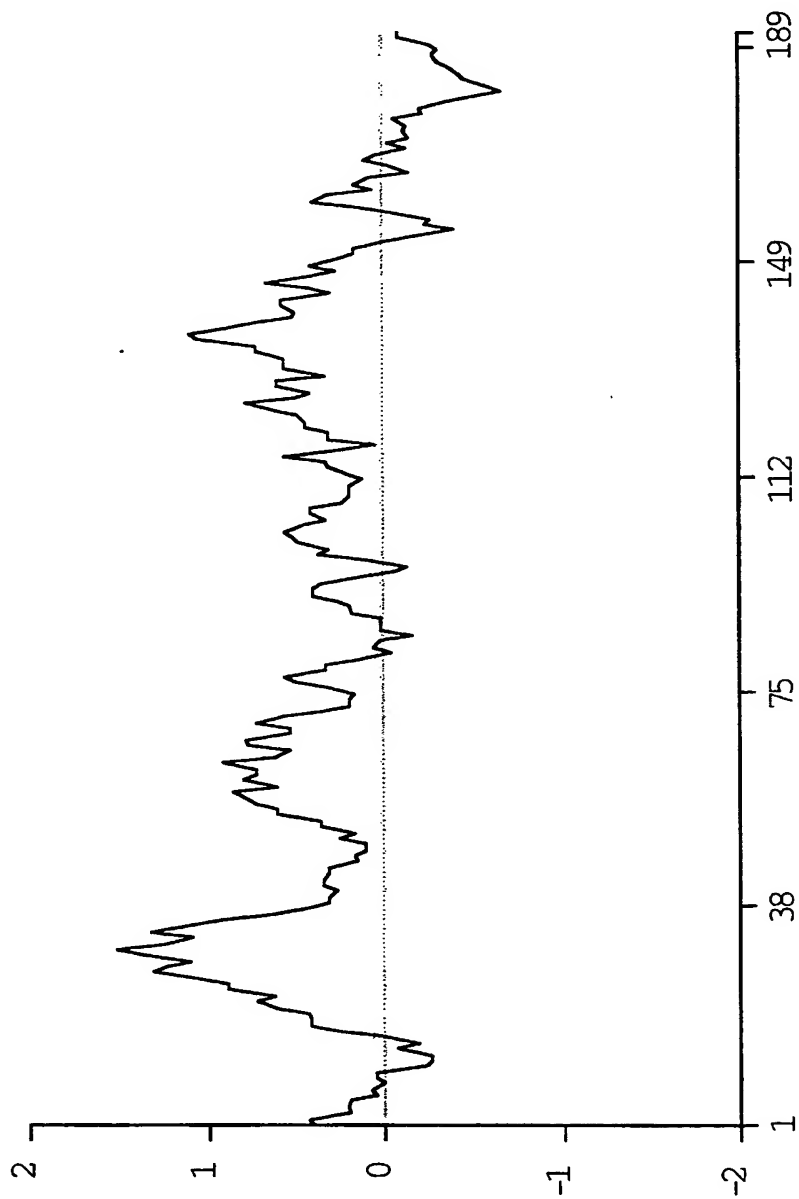


FIGURE 3B

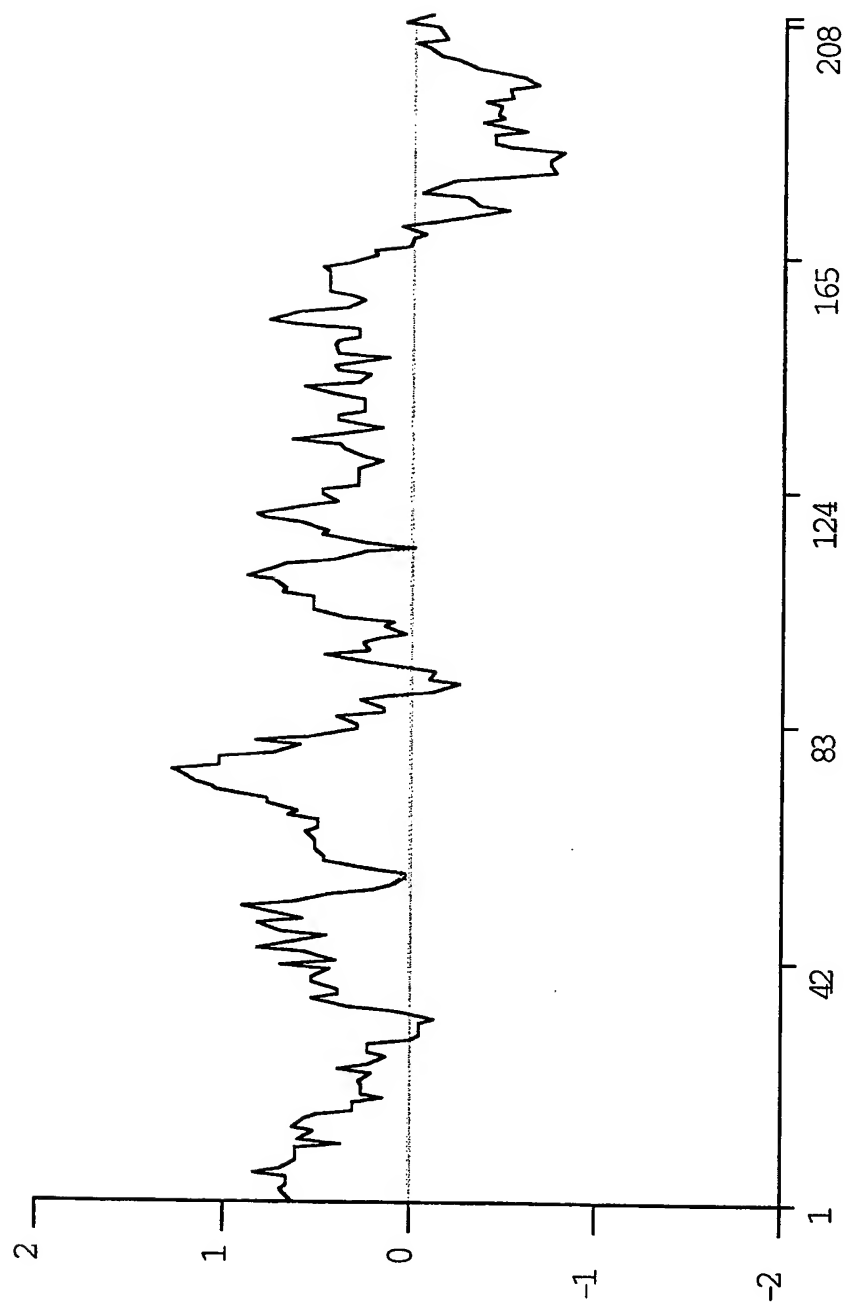


FIGURE 3C

# INTERNATIONAL SEARCH REPORT

Int. lional Application No  
PCT/US 98/13393

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC 6 C12N15/12 C07K14/47 A61K38/17 C07K16/18 C12Q1/68		
According to International Patent Classification(IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A61K C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL: 'Homo sapiens cDNA clone 308707 5' similar to SW:CANFA P10463 CALCYPHOSINE.' EMBEST Database entry HS222332; 09-May-1996, Accession number W25222 XP002082147 see sequende	4
Y	--- -/--	1-3,5-11
<div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.</span> <span><input checked="" type="checkbox"/> Patent family members are listed in annex.</span> </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search  <div style="text-align: center; font-weight: bold;">26 October 1998</div>		Date of mailing of the international search report  <div style="text-align: center; font-weight: bold;">10/11/1998</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer  <div style="text-align: center; font-weight: bold;">Espen, J</div>

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/13393

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HILLIER L ET AL: 'Homo sapiens cDNA clone 308707 5' similar to SW:CANFA P10463 CALCYPHOSINE.' EMEST Database entry HSN95268; 19-Apr-1996, Accession number N95268 XP002082148	5
Y	see sequence	1-3,5-11
Y	---- LEFORT A ET AL: 'Calcyphosine (Thyroid protein P24); Canis familiaris.' SWISSPROT Database entry CAYP_CANFA, 01-Jul-1989; Accession number P10463 XP002082149 see sequence	1-3,5-11
X	---- WO 93 25685 A (MASSACHUSETTS INST TECHNOLOGY) 23 December 1993 see SEQ ID NOs 10,13 see page 4 - page 5; figure 3 see page 39 - page 42 -----	1



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 98/13393

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 16, 17  
are directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☒ Claims Nos.: 14, 15  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:  
Said claims relate to antagonists and agonists without giving a true  
technical characterization of the claimed matter. In consequence, the scope  
of said claims is ambiguous and, moreover, their subject-matter is vague  
and not sufficiently disclosed.
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/13393

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9325685 A	23-12-1993	EP 0672151 A	20-09-1995
		JP 8500482 T	23-01-1996
		WO 9325694 A	23-12-1993
<hr/>			